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Structure, Activity, and Immune (T and B Cell) Recognition of Botulinum Neurotoxins

M. Zouhair Atassi[1,2] and Minako Oshima[1]*

[1]Department of Biochemistry and [2]Department of Microbiology and Immunology, Baylor College of Medicine, Houston, Texas 77030

* Address all correspondence to: Dr. M. Z. Atassi, Department of Biochemistry, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030

Referee: Dr. Colin R. Young, United States Department of Agriculture, Agricultural Research Service, 2881 F&B Road, College Station, TX 77845-9594

ABSTRACT: Botulism, which was first reported over a century ago, is caused by botulinum neurotoxins produced by *Clostridium botulinum* in seven immunological serotypes (A through G). The primary structures of a number of these BoNTs have been determined and are reviewed here, together with their gene structure and synthesis. The biological actions of BoNTs, which result in their ability to block neurotransmitter release have been the subject of intensive study, and in this review we discuss the binding of BoNTs to the cell surface as well as the mechanism of their intercellular action. The ability of BoNTs to block neurotransmitter release has been exploited in therapeutic applications to reduce muscle hyperactivity for the treatment of a variety of clinical conditions associated with involuntary muscle spasm and contractions. The advantages, limitations, and risks of these applications are discussed. Certain compounds provide some limited protection against BoNT. However, more effective protection has been obtained immunologically either by passive immunity (i.e., by administration of anti-BoNT Abs) or by immunization with inactivated toxin. More recently, excellent protection has been obtained by immunization with the receptor-binding region comprising the C-terminal (residues 860 to 1296) fragment (H_C) of the heavy chain of BoNT/A. Here we review the mapping of the epitopes on the H_C region of BoNT/A that are recognized by anti-BoNT/A Abs raised in horse, human, and mouse. The epitopes on the H_C that are recognized by anti-H_C Abs and by H_C-primed T lymphocytes were mapped in two mouse strains [BALB/c ($H\text{-}2^d$) and SJL ($H\text{-}2^s$)]. The peptides, which contain Ab or T cell epitopes (or both) on the H_C, were used as immunogens in BALB/c and SJL mice and we identified those peptides whose Ab and/or T-cell responses cross-react with H_C. Identification of these peptides is an important first step in the intricate requirements for the design of a synthetic vaccine.

KEY WORDS: botulinum neurotoxin, synthetic peptides, antibodies, T-cells, epitopes.

ABBREVIATIONS

Ab, antibody; **ACh**, acetylcholine; **BoNT**, botulinum neurotoxin; **BoNT/A to G**, BoNT type A, B, C, D, E, F, or G; **BSA**, bovine serum albumin; **HA**, hemagglutinin; **H_C**, C-terminal fragment corresponding to residues 855 to 1296 of the heavy chain of BoNT/A; **LNC**, lymph node cells; **mAb**, monoclonal Ab; **MHC**, major histocompatibility complex; **NTNH**, nontoxin, nonhemagglutinin components; **RIA**, radioimmune assay; **s.c.**, subcutaneous; **SD**, standard deviation; **S.I.**, stimulation index, which is mean cpm incorporated *in vitro* by stimulated T cells/mean cpm incorporated by unstimulated T cells; **SNAP**, synaptosome-associated proteins; **t-SNARE**, target-SNAP receptors complex; **v-SNARE**, vesicle membrane receptors complex; **TeNT**, tetanus neurotoxin; **VAMP**, vesicle-associated membrane protein.

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I. INTRODUCTION

Botulism due to toxin in food was first reported in 1897 by Van Ermengem.[1,2] Poisoning is caused mainly by botulinum neurotoxins (BoNTs),[3] a group of protein neurotoxins produced by *Clostridium botulinum*.[4] Seven immunological BoNT serotypes (A through G) are known of which type C has two subtypes (C1 and C2). Human botulism is most frequently caused by types A, B, and E, and rarely by type F, while animals are more often infected by types C and D.[5]

Five different forms of botulism are known:[5-7] (1) foodborne botulism, caused by ingestion of *C. botulinum*-contaminated food; (2) wound botulism, caused by infection of wounds and has also been observed increasingly in drug addicts; (3) infant botulism is the most frequent and results from ingestion of the *C. botulinum* organism and its colonization of the intestine.[8] It can also occur in adults suffering from chronic gastrointestinal disease;[7] (4) hidden botulism in adults is similar to infant botulism and occurs in cases of abnormal intestines; (5) inadvertent botulism can result after BoNT treatments for movement disorders.

Botulinum neurotoxins are the most toxic substances known.[9,10] For example on a molar basis, BoNT is 300-fold more lethal than diphtheria toxin, 3×10^4 more toxic than ricin, 3×10^6 more toxic than α-bungarotoxin, 1×10^9 more toxic than curare, and 1×10^{11} more toxic than NaCN.[10]

II. SYNTHESIS AND STRUCTURE OF BoNTS

Botulinum neurotoxins are synthesized in a progenitor toxin as a single polypeptide chain[11,12] that has a molecular weight of about 150 kDa. After secretion, it is activated by proteolytic processing that results in scission (nicking) of a single peptide bond. In *C. botulinum* strains producing BoNTs A, C, D, and some types of B and F, the proteolytic enzyme is endogenous, while other strains (type E and some types B and F) rely on an exogenous protease (e.g., trypsin) for activation.[11,13,14] The active forms of the various types of BoNT appear to have a common subunit structure.[11,13,15-21] Typically, the two subunits resulting from the nicking of the progenitor toxin have molecular weights of about 100 kDa (heavy or H) chain and 50 kDa (light or L) chain. Except in BoNT/C2, the two subunits are held together by a disulfide bond.[13,16,17,21] Reduction of the interchain disulfide bond causes loss of toxicity,[13] and the two subunits can be reassembled to reform the active toxin.[21] BoNT/C2 also has two subunits (100 kDa H-chain and 50 kDa L-chain), but they are not covalently linked.[19] The two subunits of BoNT/C2 can be separated and each alone has low toxicity but become extremely active when combined.[20,22]

Three types of progenitor toxins (19S, 16S, and 12S) are produced by *C. botulinum* type A strain (A-NIH).[23,24] The 19S and 16S toxins contain nontoxin, nonhemagglutinin (NTNH) components and an adjacent open reading frame between the neurotoxin and the hemagglutinin (HA) gene.[23] In both the 19S and 16S, the NTNH is a single peptide chain of about 120 kDa,[23] but it appears in the 19S to be a dimer of the 16S[24] and the NTNH of the 12S results from cleavage of whole NTNH.[24]

The hemagglutinin components in types B and C progenitor toxin exhibit significant homology.[25] In *C. botulinum* type A(B) strain NCTC 2916, the BoNT/A gene cluster encodes BoNT, a NTNH, and a part of P-47.[26] The gene for the latter protein is also found in *C. botulinum* types E and F. This strain also has a silent BoNT/B gene as well as genes encoding NTNH, a putative regulator gene P-21, hemagglutinin proteins HA-33,[26] HA70, and HA17, and a gene that produces a protein, OrfX, that shows homology to regulatory proteins.[27] Similar sequences were found at equivalent positions in the gene complex of tetanus neurotoxin (TeNT).[27] These proteins may be involved in coordination of the expression of the gene components of the BoNT complex and the TeNT genes.[27] The NTNH molecules have 471 amino acids and are identical in types A and B gene clusters.[26]

C. botulinum type D, strain CB-16, produces two progenitor toxins of sizes 300 kDa and 500 kDa. The NTNH of the 300-kDa toxin results from cleavage of the NTNH in a larger 500-kDa toxin at a unique Thr-Ser peptide bond.[28] The gene cluster of type E progenitor toxin is a spe-

cific arrangement (class IV) among the BoNT complex genes.[29] The gene cluster of the BoNT complex in *C. botulinum* type G reveals, immediately upstream of BoNT/G, a gene that encodes a protein of 1198 amino acids homologous to the NTNH component of the progenitor toxin.[30] Genes encoding hemagglutinin proteins (HA-17, HA-70) and a putative regulator gene (P-21) occur further upstream.[30] BoNT/G shows the highest homology to BoNT/B,[30] and NTNH of type G has the highest homology with NTNH of type B.[30]

Some *C. botulinum* type A strains show no BoNT/B activity, but they possess silent type B gene sequences that contain a stop signal and deletions.[31] In these strains, genes of HA-II and HA-33 were found immediately upstream of the silent BoNT/B but not the BoNT/A gene. NTNH mapped immediately upstream of the BoNT/A and the silent BoNT/B genes and was chimeric, having a region that is identical to NTNH of type A as well as a region that is highly homologous to the NTNH of type B.[31]

BoNTs and TeNT both block neurotransmitter release, and the mechanisms of their poisoning are very similar. However, the clinical symptoms caused by BoNTs are different from those caused by TeNT[32] and BoNT is a food poison, whereas TeNT is not. These differences have been attributed to the heavy chains of BoNTs and TeNT, which apparently use different routes for transporting the L chain to its site of action,[32] or to the production of complexing proteins by *C. botulinum* but not by *C. tetani*.[33]

The complete primary structures of BoNT/A,[34,35] B,[36,37] C1,[38,39] D,[40,41] E,[42,43] F,[44] and G[45,46] have been determined (see Figure 1). In addition, the disulfide pairing in BoNT/A has been established.[47] BoNT shows extensive homology to TeNT.[34,40,48,49] Four cysteine residues are conserved in BoNT/A and TeNT.

Neurotoxin mutants have been reported in *C. botulinum* type A.[50] Also, some strains of *C. botulinum* contain genes that encode 'mosaic' neurotoxins. For example, the genes in *C. botulinum* type C strain 6813 encode a BoNT of 1280 amino acids (mol wt. 147,817) in which the first two-thirds of its sequence is 95% identical with BoNT/C1, and the last (C-terminal) third has 95% identity with the C-terminal third of BoNT/D.[51] The gene encoding the BoNT from *C. botulinum*, type D South African (Dsa) strain, has three regions. Regions 1 to 522 and 945 to 1285 are highly homologous to the corresponding regions of BoNT/D and BoNT/C1, respectively. The central region (residues 523 to 944) is similar in the three toxins.[52] BoNT/C2 is produced by *C. botulinum* types C and D. The nucleotide sequence of the L-chain gene of *C. botulinum* type C strain ©-203U28 encodes 431 amino acid residues (49.4 kDa).[53]

Theoretical predictions were made of the channel-forming regions of BoNT heavy chain.[54] A synthetic peptide (GAVILLEFIPEIAIPVLGTFALV) that mimicked the predicted transmembrane sequence of BoNT/A has been proposed to be involved in the ion channel-forming motif.[55]

Crystallization and preliminary X-ray analysis of BoNT type A have been reported.[56] Preliminary crystallization of the translocation domain of BoNT/A has been reported recently on a recombinant preparation that was obtained by expression in *E. coli*.[57] The 900 kDa BoNT complex of serotype A has been crystallized by a lipid-layer two-dimensional crystallization technique.[58] The crystals, which diffracted to better than 15 Å in negative stain, showed a triangular core structure that has six lobes and six smaller structural protrusions.[58]

III. BIOLOGICAL ACTION OF BoNTS

A. Binding to the Cell Surface

It has long been recognized that BoNT acts on the nervous system[59,60] and causes paralysis by blockage of acetylcholine (ACh) release from nerve terminals at the neuromuscular junction.[3,4,14,61-64] In this respect, its action is quite similar to that of TeNT, which also blocks ACh release from the neuromuscular junction.[65,66] Burgen et al.[61] reported the first evidence that the action of BoNT on the neuromuscular junction involves binding to a receptor and that the binding step is distinct from the onset of toxicity. BoNT-induced blockade of neuromuscular transmission was proposed to involve sequential steps[67] in a manner similar to that described for diphtheria toxin.[68-72] The action of BoNT is initiated by the binding of BoNT to an acceptor molecule on the cell surface. The toxin-receptor

A or * = catalytic residue Z = zinc binding histidine.

FIGURE 1

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BoNT/A  285 yyyNkFKdIAStLNk akSi vgttasLQymKNVFkEKYlLseDTSGkFSVDklKFDkLY
Inft A  285 yyyNkFKdVAStLNk akSi igttasLQymKNVFkEKYlLseDTSGkFSVDklKFDkLY
BoNT/D  292 kaLghYKdIAkRLNnInkTipsswisNIDkYKkIFsEKYnfDkDnTGnFvVNiDKFNsLY
BoNT/C  292 kaLDyYRsIAkRLNsIttanpssfnkyIgeYKQklirKYrfvvESSGevTVNrNKFveLY
BoNT/B  291 kvLQNFRgIvdRLNkVlvCi sdpnININiYKNkFkDKYkfveDSeGkYSIDvEsFDkLY
BoNT/G  291 kaLQNFqdIAnRLNiV sSa qgsgIDIslYKQIYkNKYdfveDpnGkYSVDkDKFDkLY
 TeNT   294 ktLNDYKaIAnKLsqVtsCn  dpnIDIDsYKQIYqQKYqfDkDSnGqYiVNeDKFQiLY
BoNT/F  289 nlLaNYekIATRLseVnsapp   eyDINeYKDyFqwKYgLDkNadGsYTVNeNKFNeIY
ClBarF  281 ilLsNYtaIASRLsqVnrnns   aLNttyYKNfFqwKYgLDqDSnGnYTVNisKFNaIY
BoNT/E  274 nlLaDYKkIASKLskVqvSnp   lLN  pYKDVFeaKYgLDkDaSGiYSVNiNKFNdIF
ClButE  274 nlLaDYKkIASKLskVqvSnp   lLN  pYKDVFeaKYgLDkDaSGiYSVNiNKFNdIF

BoNT/A  343 KmLteiYTEdNfvkfFkVlnRkTYLnfdkavfKI NIVpkvnYTIyDGFNLrntNLaaNF
Inft A  343 KmLteiYTEdNfvnfFkVinRkTYLnfdkavfRI NIVpDEnYTIkDGFNLkgaNLstNF
BoNT/D  352 sdLtnvmSEvvyssqYNVKnRthYfsrhylPV faNILDDNIYTIrDGFNL  tN Kgfnien
BoNT/C  352 neLtqiFTEfNyAkiYNVqnRkiYLsnvytPV taNILDDNVYdIqNGFNIpksNLnvlF
BoNT/B  350 KsLmFgFTEtNIAenYkIKTRaSYfsdslpPVKIkNLLDNEIYTIeEGFNIsdkDmekEY
BoNT/G  349 KaLmFgFTEtNLAgeYgIKTRySYfseylpPIKtekLLDNtIYTqnEGFNIaskNLKtEF
 TeNT   352 nsImYgFTEiELgkkFNIKTRlSYfsmnhdPVKIpNLLDDtIYndTEGFNIeskDLKsEY
BoNT/F  346 KkL YsFTEsDLAnkFkVKCRnTYfiky efLKVpNLLDDDIYTVSEGFNIg  NLavNn
ClBarF  338 KkL FsFTEcDLAqkFQVKnRsnYLfhfk PfRLlDLLDDNIYSISEGFNIgs  LRvNn
BoNT/E  329 KkL YsFTEfDLAtkFQVKCRqTYIgqyk yfKLsNLLNDsIYnISEGYNIn  NLKvNF
ClButE  329 KkL YsFTEfDLAtkFQVKCRqTYIgqyk yfKLsNLLNDsIYnISEGYNIn  NLKvNF

BoNT/A  402 nGQNtEINnmnftkLkNftGLfeFyKLL  CvRgIITsKt ksldkgynk     448
Inft A  402 nGQNtEINsrnftrLkNftGLfeFyKLL  CvRgIIpfKt ksldegynk     448
BoNT/D  411 sGQNiErNpaLqklssEsv VdlFtKV   ClR  LT K                442
BoNT/C  411 mGQNlsrNpaLrkvnpEnm LylFtKf   ChKaI DgRs       lynk     449
BoNT/B  410 rGQNkaINkqayeeIskeh LavY KI qmC K   SvK                441
BoNT/G  409 nGQNkaVNkeayeeIsleh LViY RI amC KpV myK                442
 TeNT   412 kGQNmrVNtnafrnV DgsGLVs  KLIglC KkIIpptnir  enlynrta   457
BoNT/F  402 rGQsikLNpkIidsIpDk GLVe  KIVkfC KsVIprK                436
ClBarF  394 nGQNiNLNsrIvgpIpDn GLVe  RfVglC KSIVSkK (cleavage position unknown)
BoNT/E  385 rGQNaNLNprIitpItgr GLVk  KIIrfC KnIVSvKgir             422
ClButE  385 rGQNaNLNprIitpItgr GLVk  KIIrfC KnIVSvKgir             422
⇑Light Chains_____________________________________________________
Heavy Chains
⇓
BoNT/A  449     alndLC  IKVNNwDLFFspSEDnFTNDLnkgE EItsDTNIEaaEENi  SLD LIqQYy
Inft A  449     alndLC  IKVNNwDLFFspSEDnFTNDLdkvE EItaDTNIEaaEENi  SLD LIqQYy
BoNT/D  443   nsrddstC  IKVkNnrLpYVAdkDSiSQEIfE NKiItdETNVQnysDkF  SLDEsILDgQ
BoNT/C  450      tld CreLlVkNtDLpFIgdisdvktDIflr KDINeETEViyypDNv  SVDQVILskN
BoNT/B  442     ap gIC  IdVDNeDLFFIAdkNSFSDDLsknER IEyNTQsNyiENDFp  INELILDtD
BoNT/G  443  ntgks eqC  IiVNNeDLFFIAnkDSFSkDLakaE tIayNTQnNtiENNF  SIDQLILDnD
 TeNT   458 sltdlggeLC  IKIkNeDLtFIAekNSFSEEpfQdE iVsyNTknkplNfNY  SLDkIIVDYN
BoNT/F  437  gtkapprLC  IRVNNsELFFVASEsSYnENdiNtpKEID DT tN lNNNYrnnLDEVILDYN
ClBarF  429  gtk  nsLC  IKVNNrDLFFVASEsSYnENgiNspKEID DTtIt  NNNYkknLDEVILDYN
BoNT/E  423       ksIC  IeINNgELFFVASENSYnDDniNtpKEID DT Vts NNNYendLDQVILNFN
ClButE  423       ksIC  IeINNgELFFVASENSYnDDniNtpKEID DT Vts NNNYendLDQVILNFN

BoNT/A  504 LtfnfDnepEnIsienlssdI IgqlelmP  NiErfpNgKkyE LDkyt mFhYLrAQefe
Inft A  504 LtfdfDnepEnIsienlssdI IgqlepmP  NiErfpNgKkyE LDkyt mFhYLrAQefe
BoNT/D  501 VpinpEIvDplLpnvnmEp LnL      PgeEivfydDItky  VD ylNsYYYLesQKls
BoNT/C  506 tsehgQL DllypsidsEseI L      PgeN QvfyDnRtqN VD ylNsYYYLesQKls
BoNT/B  497 LiSkiELpsEntEsltDfN VdV      PvYEkQ  paIKkif tDent IFqYLysQtfP
BoNT/G  501 LsSgiDLpNEntEpftNfDdIdI      PvYikQ  SaLKkif VDgds LFeYLhAQtfP
 TeNT   518 LqSkitLpNDrttpvtkgi pya      PeYkSNaaStIeihN IDdnt IYqYLyAQKsP
BoNT/F  496 sqTipQIsNrtLNtlvQDN syV      PrYDSNgtSEIeeyDvVD f NVFFYLhAQKvP
ClBarF  486 sdaipNLssrlLNttaQND syV      PkYDSNgtSEIKeyt VDk lNVFFYLyAQKaP
BoNT/E  477 seSapgLsDEkLNltiQND ayI      PkYDSNgtSDIeqhD VNe lNVFFYLdAQKvP
ClButE  477 seSapgLsDEkLNltiQND ayI      PkYDSNgtSDIeqhD VNe lNVFFYLdAQKvP
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FIGURE 1 (continued)

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BoNT/A  561 hGksrIaLTnSVNEALLNpsRVYTFFSSDYVkKVNKatEAamFLgWVEQLVyDFTdEtsEvS
Inft A  561 hGdsrIiLTnSaEEALLkpnvaYTFFSSkYVkKINKaVEAfmFLnWaEELVyDFTdEtnEvT
BoNT/D  553 NnyENITLTTSVEEALgysnKIYTFlpS laEKVNKgVQAgLFLnWaNEVVEDFTTNimkK d
BoNT/C  557 DnyEDfTfTrSIEEALdNsaKVYTYFpT laNKVNagVQggLFLmWaNDVVEDFTTNilrK d
BoNT/B  548 ldirDISLTSSfDDALLfsnKVYSFFSmDYIktaNKvVEAgLFagWVkQIVNDFviEa NKSn
BoNT/G  553 sniENLqLTnSLNDALrNnnKVYTFFSTNlVEKaNtvVGAsLFVnWVkgVIDDFTSEstQKS
 TeNT   571 ttlQrITmTnSVDDALINstKIYSYFpS vIsKVNqgaQgiLFLqWVrDIIDDFTnEssQKT
BoNT/F  549 EGetNISLTSSIDtALLEesKd iFFSSEFIDtINKpVNAaLFIdWIskVIrDFTTEatQKS
ClBarF  539 EGesaISLTSSVNtALLDasKVYTFFSSDFINtVNKpVQAaLFIsWIQQVINDFTTEatQKS
BoNT/E  530 EGeNNVnLTSSIDtALLEqpKIYTFFSSEFINnVNKpVQAaLFVsWIQQVLvDFTTEanQKS
ClButE  530 EGeNNVnLTSSIDtALLEqpKIYTFFSSEFINnVNKpVQAaLFVgWIQQVLvDFTTEanQKS

BoNT/A  623 TtDKIADITIIIPYIGPALNIGNmlyKdDFvgALifsGAvILLEFIPEIaIPVLGtFa
Inft A  623 TmDKIADITIIVPYIGPALNIGNmlsKGEFvEAIiftGvvamLEFIPEyaLPVfGtFa
BoNT/D  614 TLDKIsDVSvIIPYIGPALNIGNSalRGNFnQAfataGvafLLEgfPEftIPaLGvFT
BoNT/C  618 TLDKIsDVSaIIPYIGPALNIsNsvrRGNFtEAfaVtGvtILLEafPEftIPaLGaFv
BoNT/B  610 TmDKIADISLIVPYIGlALNVGNEtaKGNFeNAfEIaGAsILLEFIPELlIPVVGaFl
BoNT/G  615 TIDKVsDVSIIIPYIGPALNVGNEtaKeNFkNAfEIgGAaILmEFIPELiVPIVGfFT
 TeNT   632 TIDKIsDVStIVPYIGPALNIvkQgyeGNFigALEttGvvLLLEYIPEItLPVIaalS
BoNT/F  610 TVDKIADISLIVPYVGlALNIiiEaeKGNFeEAfELlGvgILLEFVPELtIPVIlvFT
ClBarF  601 TIDKIADISLIVPYVGlALNIGNEvqKGNFkEAIELlGAgILLEFVPELlIPtIlvFT
BoNT/E  592 TVDKIADISIVVPYIGlALNIGNEaqKGNFkDALELlGAgILLEFePELlIPtIlvFT
ClButE  592 TVDKIADISIVVPYIGlALNIGNEaqKGNFkDALELlGAgILLEFePELlIPtIlvFT

BoNT/A  681 LvSYIa    NKvltVqTIDNALskRNEKWdEVYkYIVTNWLaKVNTQiDlIRkkMkEALE
Inft A  681 IvSYIa    NKvltVqTINNALskRNEKWdEVYkYtVTNWLaKVNTQiDlIREkMkkALE
BoNT/D  672 fySsIq    ERKIIKTIENcLeQRvkRWKDsYqWmVSNWLSRItTQFNhInyQMYDsLs
BoNT/C  676 IySkVq    ERNeIIKTIDNcLeQRikRWKDsYeWmmgtWLSRIiTQFNnIsyQMYDsLN
BoNT/B  668 LeSYId    NKNKIIKTIDNALtkRNEKWsDmYglIVaQWLStVNTQFytIKEgMYkALN
BoNT/G  673 LeSYVg    NKghIImTIsNALkkRDqKWtDmYglIVSQWLStVNTQFytIKErMYNALN
 TeNT   690 Iaess t   QKEKIIKTIDNfLekRyEKWiEVYklVkakWLgtVNTQFQkrsyQMYrsLE
BoNT/F  668 IkSYIdsyeNKNKaIKaINNsLiERRaKWKEIYsWIVSNWLTRINTQFNkrKEQMYQALQ
ClBarF  659 IkSFInsddsKNKIIKaINNALrERElKWKEVYsWIVSNWLTRINTQFNkrKEQMYQALQ
BoNT/E  650 IkSFLgssdNKNKVIKaINNALkERDEKWKEVYsFIVSNWmTKINTQFNkrKEQMYQALQ
ClButE  650 IKSFLgssdNKNKVIKaINNALkERDEKWKEVYsFIVSNWmTKINTQFNkrKEQMYQALQ

BoNT/A  738 NQaEAtKaIINYQYNqYTeEEKNNI  NFNIDDLsskLNEsInkAMiNINKFLnQCSVSY
Inft A  738 NQaEAtKaIINYQYNqYTeEEKNNI  NFNIDDLsskLNEsInsAMiNINKFLdQCSVSY
BoNT/D  729 yQaDAIKakIDlEYkkYSgsDKENIk  sQVENLkNsLDvKISeAMNNINKFIrECSVTY
BoNT/C  733 yQagAIKakIDlEYkkYSgsDKENIk  sQVENLkNsLDvKISeAMNNINKFIrECSVTY
BoNT/B  725 yQaQALeeIIkYRYNiYSekEKsNI  NiDfNDInskLNEgInqAiDNINnFIngCSVSY
BoNT/G  730 NQsQAIekIIEdQYNrYSeEDKmNI  NiDfNDIdfkLNQsInlAiNNIDdFInQCSISY
 TeNT   747 yQvDAIKkIIDYEYkiYSgpDKEQIa D EINNLkNkLEEKankAMiNINiFmrESSrSF
BoNT/F  728 NQvDAIKtaIEYkYNnYTsDEKNrLesEYNINNIeEeLNkKVSlAMkNIERFmtESSISY
ClBarF  719 NQvDgIKkIIEYkYNnYTlDEKNrLraEYNIysIkEeLNkKVSlAMQNIDRFLtESSISY
BoNT/E  710 NQvNAIKtIIEskYNsYTlEEKNELtnkYDIkQIeNeLNQKVSiAMNNIDRFLtESSISY
ClButE  710 NQvNALKaIIEskYNsYTlEEKNELtnkYDIEQIeNeLNQKVSiAMNNIDRFLtESSISY

BoNT/A  796 LMnsMIPyg VkRLeDFDasLKdaLLkYIyDNrgtLIgQv DrLKdKVNNTLstdIPFQLS
Inft A  796 LMnsMIPyA VkRLkDFDasVRdvLLkYIyDNrgtLVlQv DrLKdeVNNTLsadIPFQLS
BoNT/D  787 LfKnMLPkv IDeLnkFDlrtKteLINlIdshniiLVgEv DrLKaKVNNSfQNTIPFNIf
BoNT/C  791 LfKnMLPkv IDeLnEFDrntKakLINlIdshniiLVgEv DkLKaKVNNSfQNTIPFNIf
BoNT/B  783 LMKkMIPlA VEKLlDFDntLKknLLNYIdENklyLIgsa EyeKsKVNkyLktimPFDLS
BoNT/G  788 LMnrMIPlA VkKLkDFDdnLKrdLLEYIdtNelyLLdEv NiLKsKVNrhLkDSIPFDLS
 TeNT   805 LvnqMIneAk kqLlEFDtqsKniLmQYIkaNskfIgitelkkLesKINkvfstpIPF
BoNT/F  788 LMK lIneAkVgKLkkYDnhVKsdLLNYIlDhrsiLgeQt NeLsdlVtsTLNsSIPFELS
ClBarF  779 LMK lIneAkINKLsEYDkrVnqyLLNYIlENsstLgtssvpeLnnlVsNTLNNSIPFELS
BoNT/E  770 LMK lInevkINKLrEYDenVKtyLLNYIiQhgsiLgesq QeLnsmVtDTLNNSIPFkLS
ClButE  770 LMK lInevkINKLrEYDenVKtyLLDYIikhgsiLgesq QeLnsmViDTLNNSIPFkLS
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FIGURE 1 (continued)

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BoNT/A  855 kYvDNqrLLs tFtEYIKnIiNTSILNLRYesNhLIDlSrYaSkINIgskVnfDpiDKNQ
Inft A  855 kYvDNkkLLs tFtEYIKnIvNTSILsIvYKkDdLIDlSrYgakINIgdrVyyDsiDKNQ
BoNT/D  846 SYTNNsLLkd iiNEYFnsInDSkILsLqnKkNaLVDTSGYnaEVrVgdNVqLNtiytND
BoNT/C  850 SYTNNsLLkd iiNEYFnnInDSkILsLqnRkNtLVDTSGYnaEVseEgDVqLNpifpfD
BoNT/B  842 iYTNDtILIe mFNkYnseIlNniILNLRYKDNnLIDlSGYgakVEVydgVeLNd  KNQ
BoNT/G  847 lYTkDtILIq vFNNYisnIssnaILsLsYRggrLIDSSGYgatmNVgsDVifNdigngQ
 TeNT   862 SYSkNldcwvdneEDidviLkkSTILNLdinNDiIsDiSGFnSsVitypDaqLvpging
BoNT/F  847 SYTNDkILIi yFNrlYKkIkDSSILDmRYeNNkfIDiSGYgSNIsINgNVyIystNRNQ
ClBarF  839 eYTNDkILIh ilirFYKrIiDSSILNmKYeNNrfIDSSGYgSNIsINgDIyIystNRNQ
BoNT/E  829 SYTDDkILIs yFNkFFKrIksSSVLNmRYKNDkyVDTSGYdSNININgDVykyptNKNQ
ClButE  829 SYTDDkILIs yFNkFFKrIksSSVLNmRYKNDkyVDTSGYdSNININgDVykyptNKNQ

BoNT/A  914 iqLfN  LEs SkIEVilkNaIVYNSmYENFStSFWIRIPK YfNs Is  LNNEYTIIN
Inft A  914 iKLiN  LEs StIEVilkNaIVYNSmYENFStSFWIKIPK Yfsk I N LNNEYTIIN
BoNT/D  905 FKLssSg Dk   IiVnlNNNILYsaiYENsSVSFWIKIsKdltNsh      NEYTIIN
BoNT/C  909 FKLgsSg Edrgk ViVtQNENIVYNSmYEsFSISFWIRInK Wvsn L     pgYTIID
BoNT/B  899 FKLtsS aN  SkIrVtQNQNIIFNSvFlDFSVSFWIRIPK YkNdgIqNyIhNEYTIIN
BoNT/G  906 FKLnNS  En SNItahQskfVVYDSmFDNFSInFWVRtPK YNNndIqtyLQNEYTIIs
 TeNT   921  KaihlvnNesSEViVhkamDIeYNdmFNNFTVSFWLRVPK vsashLeQygtNEYSIIs
BoNT/F  906 FgIyNSrL   SEVNIaQNNDIIYNSrYQNFSISFWVRIPKhYk   pmN hNrEYTIIN
ClBarF  898 FgIyssSrL   SEVNItQNNtIIYNSrYQNFSVSFWVRIPK YNN  LkN LNNEYTIIN
BoNT/E  888 FgIyNdkL   SEVNIsQNDyIIYDnkYkNFSISFWVRIP nYDNk IvN VNNEYTIIN
ClButE  888 FgIyNdkL   SEVNIsQNDyIIYDnkYkNFSISFWVRIP nYDNk IvN VNNEYTIIN

BoNT/A  967 CM EN    NSGWKVSLny   gEIIWTLQDtqeikQrVvFkYsQmiNISDYI NRWIFV
Inft A  967 Ci EN    NSGWKVSLny   gEIIWTLQDnKqniQrVvFkYsQmvNISDYI NRWIFV
BoNT/D  955 Si EQ    NSGWKLCIrn   gNIeWiLQDvnrkyksLiFDYsEslshtgYT NKWFFV
BoNT/C  961 Sv kN    NSGWsIgIis   NfLVFTLkqnedseQsInFsYdisNNapgY  NKWFFV
BoNT/B  955 CM kN    NSGWKISIrg   NrIIWTLiDinGktksVfFEYnirEDISEYI NRWFFV
BoNT/G  962 Ci kN    DSGWKVSIkg   NrIIWTLiDvnaksksIfFEYsikDNISDYI NKWFsI
 TeNT   979 SMkkhslsigSGWsVSLkg   NNLIWTLkDsaGevrqItFr dlpDkfnaYLaNKWVFI
BoNT/F  959 CMgNN    NSGWKISLrtvrdcEIIWTLQDtsGnkenLiFrYeElNrISNYI NKWIFV
ClBarF  951 CMrNN    NSGWKISLny   NNIIWTLQDttGnnQkLvFNYtQmiDISDYI NKWtFV
BoNT/E  942 CMrDN    NSGWKVSLnh   NEIIWTLQDnrGinQkLaFNYgNaNgISDYI NKWIFV
ClButE  942 CMrDN    NSGWKVSLnh   NEIIWTLQDnsGinQkLaFNYgNaNgISDYI NKWIFV

BoNT/A 1018 TITNNRLnNSKIYINGrLIDQKpIsNLGNIH       aSNNIm FKLdgCrDthRYIwI
Inft A 1018 TITNNRLtkSKIYINGrLIDQKpIsNLGNIH       aSNkIm FKLdgCrDprRYImI
BoNT/D 1006 TITNNimGymKLYINGeLkQsqkIeDLdEVk       ldktIV FgIdeniDenqmLwI
BoNT/C 1011 TVTNNmmGNmKIYINGkLIDtikVkELtgInfsktitfeiNkIpdtgLItSdsdninmwI
BoNT/B 1006 TITNN LnNaKIYINGkLesNtdIkDIrEVi       angEII FKLdgdiDrTqFIwm
BoNT/G 1013 TITNDRLGNanIYINGsLkksekIlNLdrIn       sSNDI dFKLInCtDtTKFVwI
 TeNT  1035 TITNDRLssanLYINGvLmgsaeItgLGaIr       edNNIt lKLdrCnNnnqYVsI
BoNT/F 1014 TITNNRLGNSRIYINGnLIvEKsIsNLGDIH       vSDNIL FKIVgCdDeT YVgI
ClBarF 1003 TITNNRLGhSKLYINGnLtDQKsIlNLGNIH       vdDNIL FKIVgCnD TRYVgI
BoNT/E  994 TITNDRLGDSKLYINGnLIDQKsIlNLGNIH       vSDNIL FKIVnCsy TRYIgI
ClButE  994 TITNDRLGDSKLYINGnLIDQKsIlNLGNIH       vSDNIL FKIVnCsy TRYIgI

BoNT/A 1070  KYFNLFDKELNekEIkdLYdnQsNSgILKDFWGDYLqYDKpYYmLNLyd PNkYVD vnN
Inft A 1070  KYFNLFDKELNekEIkdLYdsQsNSgILKDFWGNYLqYDKpYYmLNLfd PNkYVD vnN
BoNT/D 1058  RdFNIFsKELsneDINiVYegQilrNVIKDYWGNpLkFDtEYYIIN      DnYIDR
BoNT/C 1071  RdFyIFaKELDgkDINiLFnslqyTNVVKDYWGNdLrYNKEYYmVN i    D YLNR
BoNT/B 1057  KYFsIFNtELsqSNIErYkiQsySEyLKDFWGNpLmYNKEYYmfNagnk NsYI Kl
BoNT/G 1065  KdFNIFgRELNaTEVssLYwiQssTNtLKDFWGNpLrYDtQYYLfNqgmq NiYI Ky
 TeNT  1087 dK FrIFcKaLNpkEIEkLYtsylsitfLRDFWGNpLrYDtEYYLI     PvasssKdvql
BoNT/F 1065  RYFkVFNtELDkTEIEtLYsnEpDpsILKNYWGNYLlYNKkYYLfNLlrk DkYIt  lN
ClBarF 1054  RYFkIFNmELDkTEIEtLYhsEpDStILKDFWGNYLlYNKkYYLLNLl kPNmsVt  kN
BoNT/E 1045  RYFNIFDKELDeTEIQtLYsnEpNTNILKDFWGNYLlYDKEYYLLNVl kPNnFIDRrkD
ClButE 1045  RYFNIFDKELDeTEIQtLYnnEpNaNILKDFWGNYLlYDKEYYLLNVl kPNnFINRrtD
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FIGURE 1 (continued)

FIGURE 1. Alignments of the amino acid sequences of clostridial neurotoxins. Regions of homology are indicated in boldface type, while the other regions are shown in small grey letters. Residues that are homologous in six or more proteins are shown in boldface capital letters. Residues that are homologous in five proteins (or in four proteins where, due to the introduction of gaps, the sequences of only four proteins appear in that region) are presented in boldface small letters. Inf A, infant BoNT/A, ClBar F, *Clostridium barati* neurotoxin F; ClBut E, *Clostridium butrycium* neurotoxin E. Residue marked by an asterisk (*) indicate that the residue is absent in another sequence. (For references, see the text.)

complex then undergoes endocytosis and, once inside the cell, the internalized toxin blocks neurotransmitter release.

In early studies, there was some evidence that gangliosides might be at least part of the receptor to which BoNT binds.[73] Ganglioside GT1b was

found to be effective at inactivating BoNT.[74] BoNTs A, B, E, and F were markedly inactivated by ganglioside GT1b, while BoNTs C and D suffered only mild inactivation.[75] In binding studies, it was found that different optimal conditions control the binding of gangliosides,[76,77] cerebrosides, and free fatty acids[77] to different BoNT serotypes. These findings were interpreted to mean that gangliosides may not be the common receptor for all types of BoNT,[76] and that each BoNT binds to a type-specific site on the neuronal membrane.[77] Synaptosome-incorporation experiments provided additional evidence in support of gangliosides as being part of the BoNT binding site.

It has been shown that BoNT binds to the synaptosome fraction from rat brain,[78–81] mouse;[82] *Torpedo* electric organ,[83] and rat central nervous system.[84] Using ^{125}I-labeled BoNT, the acceptor molecule was localized in murine neuromuscular junction at motor nerve terminals.[85] Electron microscope autoradiographic studies showed that there are distinct membrane acceptors on motor nerves for different types of BoNT,[86] that internalization of ^{125}I-labeled BoNT is acceptor mediated, and that the binding to cell-surface acceptors involves the H chains.[86] The BoNT uptake was energy and temperature dependent and accelerated by nerve stimulation. These studies indicated that BoNT inhibits release of ACh by interaction with an intracellular target. Neuraminidase treatment of rat brain synaptosomes impaired their ability to bind BoNT, but the binding capacity was restored by incorporation of gangliosides into these neuraminidase-treated synaptosomes.[87] A BoNT/B receptor protein has been purified 340-fold from rat synaptosomes.[81] The affinities of ^{125}I-labeled BoNT/B binding to lipid vesicles containing the receptor reconstituted with ganglioside GT1b or GD1a were the same as its binding to the native receptor on synaptosomes. Cross-linking of ^{125}I-labeled BoNT/B to the reconstituted receptor gave, under reducing conditions, a 161-kDa product. The cross-linking was inhibited by excess unlabeled BoNT/B. The cross-linked product reacted with both a monoclonal Ab (mAb) against the purified 58-kDa receptor and a mAb against the H-chain of BoNT/B. Determination of a partial amino acid sequence of the 58-kDa protein showed it to be identical to synaptotagmin (a synaptic vesicle membrane protein). The mAb against the 58-kDa receptor reacted with recombinant rat synaptotagmin. It was suggested that synaptotagmin in association with ganglioside GT1b or GD1a may be a natural receptor for BoNT/B at the nerve terminals.[81]

BoNTs A, B, or E bind to synapsin I and beta adducin (a 116-kDa bovine brain synaptosomal protein). This binding takes place through the carboxy-terminal region of the latter, and it is increased with ganglioside GT1b.[88] BoNT/A binds to (and is inactivated by) gangliosides at low, but not at high, ionic strength.[89] Using BoNT/A, it was proposed that synaptotagmin II is the molecule involved in transmitter release at mouse motor nerve terminals.[90] The binding of BoNT/B to synaptotagmin II is very low and is substantially enhanced after treatment with gangliosides GT1b or GD1a.[91,92] The binding site for BoNT/B is formed by the association of these specific gangliosides with the N-terminal region of synaptotagmin II.[91,92] These studies show that BoNT binds to synaptosomes and undergoes acceptor-mediated endocytosis and that different types of BoNT bind to different acceptors.

The binding of BoNTs A and B to synaptosomes appears to be a function of the H chain.[13,81,87,93,94] Mild trypsin action on BoNT increased the toxicity of type B 2- to 3-fold[13] and of type E 90-fold.[95] In contrast, limited trypsin treatment of BoNT/A caused loss of toxicity, which was accompanied by loss of binding to rat brain synaptosomes.[96] This treatment caused cleavage almost in the middle of the H chain giving a 46-kDa C-terminal fragment (H_C) and a 49-kDa N-terminal fragment that remained attached to the L chain by the interchain S–S bond (i.e., a 101-kDa fragment). The latter did not bind to synaptosomes, and it was suggested that the toxin-binding site resided in the C-terminal half of the H chain.[96] A similar 101-kDa fragment obtained by action of papain on BoNT/B was inactive.[87] Although binding is due to the H chain, the L chain is required for intracellular activity. It is now well established that the H chain binds to the acceptor, thereby allowing the L chain, or a combination of H and L chains, to be internalized and cause paralysis.

In summary, the H chain, which has a binding domain in the C-terminal half and a translocation domain in the N-terminal half, enables BoNT to

bind to, and penetrate, the cell surface. This permits the delivery of the L chain into the cell. The L chain is a zinc endopeptidase that has one zinc atom per molecule in all the BoNTs, except for BoNT/C, which has two zinc atoms per molecule of neurotoxin.[97] The Zn^{2+} atom(s) bound by the L chains (are) essential for its activity.[98-102] Removal of the zinc (by EDTA) causes tertiary structural changes as well as loss of the biological activity that are both irreversible.[103]

B. Intracellular Action

Following their endocytosis, BoNTs block neurotransmitter release by the proteolytic action of the L chain, which is specific for three key synaptosome-associated proteins (SNAP): synaptobrevin or VAMP (vesicle associated membrane protein), SNAP-25, and syntaxin I. These three proteins play an essential role in vesicle exocytosis from nerve terminals and neuroendocrine cells.[104-108] VAMP is an intrinsic protein of the synaptic vesicle membrane receptors complex (v-SNAREs), while SNAP-25 and syntaxin are integral plasma membrane proteins and are part of the group of proteins known as target-SNAP receptors complex (t-SNAREs) that participates in vesicle exocytosis.[109] VAMP, SNAP-25, and syntaxin are evolutionarily conserved. The BoNTs interact with a region having a nine-residue structural motif that is present in the three proteins as well as with a cleavage site on each protein.[57,98-102] VAMP contains two copies of this motif,[110,111] V1 and V2, and both are involved in the interaction with TeNT and with BoNTs B, G, and F,[111] while V1 is involved in the recognition by BoNTs D and F.[111] Abs against this motif cross-react with the three proteins and inhibit the proteolytic activity of BoNTs B and G.[110]

SNAP-25 is cleaved by BoNTs A and E. BoNT/A causes scission of the bond between residues 197 and 198, thus removing a nine-residue segment from the carboxyl terminus of SNAP-25.[112,113] BoNT/E cleaves SNAP-25 between residues 180 and 181.[114] BoNT/C1 also cleaves SNAP-25 near its C-terminus only in intact cells, but has been reported to have no action on soluble recombinant SNAP-25.[115] BoNTs B, D, F, and G (and also TeNT) effect cleavage of VAMP at a single peptide bond that is different for each toxin. Residues that are N-terminal to the site of scission on VAMP determine the endopeptidase specificity of BoNT.[116] VAMP is cleaved more effectively by recombinant light chain of BoNT/B or by trypsin-treated, reduced BoNT/B than by native BoNT/B.[117] BoNT/C causes a cleavage in syntaxin I[57,98-102,118] when inserted into a lipid bilayer.[97] BoNT/C cleaves syntaxin 1A between Lys 253 and Ala 254 and Syntaxin 1B between Lys 252 and Ala 253.[97] Syntaxin cleavage by BoNT/C prevents G-protein regulation of calcium channels associated with presynaptic neurotransmitter release sites.[119] Calcium influx through these ion channels stimulates the release of neurotransmitter into the synapse.[119]

Docking of synaptic vesicles to the presynaptic plasma membrane, which is necessary for neurotransmitter release, is followed by a fusion step that is triggered by calcium.[120] BoNTs decrease the Ca^{2+} sensitivity of the exocytotic apparatus.[121] SNAP-25 that has been cleaved by BoNT/A near the C-terminus behaves normally in the formation or the disassembly of the synaptosomal fusion complex.[113,122] Cleavage of Syntaxin by BoNT/C1 has no effect on the formation of synaptic vesicles, their number, or their distribution at the presynaptic zone, but it blocks neurotransmitter release[123,124] because it interferes with the fusion of the vesicle.[124] Docking can take place when VAMP or syntaxin are cleaved. This has been attributed to an alternative interaction of VAMP and synaptotagmin with SNAP-25 on the plasma membrane and suggested that two species of v-SNAREs (VAMP and synaptotagmin) and two species of t-SNAREs (SNAP-25 and syntaxin) interact in the docking of the synaptic vesicle.[120] Action of the L chain of BoNT/D on VAMP inhibits both Ca^{2+}-dependent and Ca^{2+}-independent neurotransmitter release.[125] BoNT/A inhibits Ca^{2+}-activated vesicle exocytosis only slightly, whereas BoNT/E causes complete inhibition.[114] This suggested that the region 181 to 197 of SNAP-25 is required for Ca^{2+}-activated membrane fusion in late postdocking events.[114] Residues 197 to 205 at the C-terminus of SNAP-25 are required for exocytosis from intact cells, whereas the region 180 to 196 is implicated in the exocytotic response of permeabilized cells and in a late MgATP-independent step of exocytosis that is

not susceptible to BoNT/A.[126] A 20-residue peptide that contains the BoNT/A-cleavage sequence and mimics the C-terminus of SNAP-25 has been shown to inhibit vesicle docking.[127] Phosphorylation of SNAP-25 at Ser-187 may be involved in protein kinase C-mediated regulation of neurotransmitter release.[128]

IV. DRUG THERAPY AGAINST TOXIN POISONING

Certain compounds show a protective activity against the paralytic actions of BoNTs. For example, drugs that elevate intraterminal free calcium or improve calcium influx (e.g., 4-aminopyridine, tetraethylammonium, guanidine, the calcium ionosphere A-23187, serotonin and quabain) will increase neurotransmitter release and act as antagonists of BoNT/A only.[129-131] The potassium channel inhibitor 3,4-diaminopyridine has been reported to act *in vitro*, on rat diaphragm muscle, as an antagonist of BoNT/A-induced paralysis.[132] Its inhibitory action on the paralysis of the rat extensor digitorum longus muscle by local injection of BoNTs A, B, E, or F showed that it is beneficial against BoNTs A and E but is marginally effective against BoNTs B and F.[133] Lysosomotropic amines (e.g., chloroquine) protect against BoNT activity[93] in a similar manner to their protection against diphtheria toxin[134,135] and pseudomonas A exotoxin.[136] Aminoquinolines act *in vitro* as pharmacological antagonists by prolonging the time to obtain 50% blockage after BoNT/A-poisoning of nerve-elicited muscle twitches in isolated mouse diaphragms.[137] The reported effectiveness of these agents, in decreasing order, is quinacrine > amodiaquine > chloroquine > quinine or quinidine.[137] Their abilities to antagonize the paralytic actions of BoNT do not seem to correlate well with their antimalarial activity.[138] Lectins from *triticum vulgaries* and *limax flavus*, two sialic acid-binding lectins, have been reported to be broad antagonists of BoNT serotypes as well as TeNT.[139] They were not tested *in vivo*, but *in vitro* they delayed paralysis time only 20 to 50 min. Because sialic acid is so ubiquitous in various tissues, it is unlikely that these or other sialic acid-binding lectins will be useful *in vivo* against BoNT. The zinc-dependent metalloprotease inhibitor, phosphoramidon, has been reported to have a significant ability *in vitro* to delay the onset of muscle twitch tension caused by action of BoNTs A or B on the mouse phrenic-nerve diaphragm.[140] The heavy metal chelator *N,N,N',N'*-tetrakis (2-pyridylmethyl)-ethylenediamine (TPEN) has been reported recently to protect mice *in vivo* by delaying BoNT/A or B lethal action, but its acute toxicity limits its usefulness *in vivo*.[141] It was suggested that TPEN, if employed in low doses, might be potentially useful in a combination therapy.[141]

Drug antagonists have a limited advantage *in vivo*, usually delaying the onset of paralysis by 1 to 2 h.[129-130] The aforementioned studies serve to indicate that no drug is presently available that could in fact counter the lethal effect of BoNT poisoning. This would emphasize the need to develop a protection strategy that is based on a detailed knowledge of the molecular and cellular immune recognition of the BoNT molecule.

V. THERAPEUTIC APPLICATIONS

The ability of BoNTs to block neurotransmitter release has been employed in minute doses (less than 1 ng), in symptoms where it is desirable to obtain a reduction of muscle hyperactivity, to produce a reversible partial paralysis at the neuromuscular junction. BoNTs have been applied, often with good results, in the treatment of a variety of clinical conditions associated with involuntary muscle spasm and contractions.[142-152] These have included various forms of dystonia,[153-157] disorders of the alimentary tract,[158-160] amyotropic lateral sclerosis,[161] dermatological and cosmetic uses,[162-169] various types of tremors and neuromyotonia,[170-176] spasticity,[147,177-179] clinical ophthalmology,[180-185] cerebral palsy,[145,186-188] disorders of anal sphincter,[189-193] urethral dilatation,[194] otorhinolaryngology,[195,196] tardive dyskinesia,[197] stiff-person syndrome,[198] adult strabismus,[199] gustatory sweating of the neck (Frey's syndrome),[200-204] focal hyperhidrosis,[205] and esophageal motor disorders.[206]

Results obtained with the injection of BoNT are not permanent and require periodic injections of the neurotoxin. Furthermore, the treatment often leads to the appearance of Ab responses against

the toxin, which render further treatment less effective.[207] This difficulty has been overcome by using another BoNT serotype that will not be neutralized by the Abs against the first BoNT that was employed in the therapy. For example, when BoNT/A was used in patients with focal dystonia, some patients mounted Ab responses against BoNT/A and became unresponsive to further treatment with BoNT/A but showed improvement that was sustained for three additional injections of BoNT/F.[208] Clearly, this strategy would not resolve the problem, and the recipient did in fact mount immune responses against the second BoNT. Increasing the BoNT dose is risky and obviously it will not resolve the problem either, because it would simply boost the Ab titer. In these treatments, lowering the BoNT dose has been recommended.[209] Studies have screened only for Ab responses,[207-209] but the results could not be explained on the basis of Ab titer only, most likely due to the presence of anti-BoNT T cell responses, which were not investigated.

It is evident that a rational application of BoNT therapy requires detailed knowledge of the submolecular structural features involved in toxin function as well as those involved in its molecular and cellular immune recognition.

VI. IMMUNE RECOGNITION OF BOTULINUM NEUROTOXINS

An immunological approach provides a more effective means for protection against BoNT. For use as an antigen in the preparation of currently-used toxoid, BoNT is usually treated for about 7 days with formaldehyde (which renders it non-toxic) and injected in horse. Protection by passive immunity requires proper diagnosis and the rapid access to an antitoxin. Because the latter is not always possible, active immunization will obviously offer a permanent and more secure protection. Reversion of formaldehyde-treated BoNT to toxicity might occur on standing, and this has been reported for tetanus and diphtheria toxoids.[210,211] Although this might be minimized or overcome by storing the toxoid in formaldehyde, such a prolonged exposure causes drastic chemical and immunological changes in proteins.[212] Antibodies against the H and L chains of BoNTs B and E showed neutralizing activity.[87,213] Some mAbs against the H chain of BoNT/E possessed a neutralizing activity.[87,214,215] Experiments *in vivo* and *in vitro* indicate that Abs can enter cholinergic nerves and neutralize internalized BoNT.[215] This is an important finding because it shows that some Abs can first act extracellularly by interfering with the binding of BoNT to the cell surface, while other Abs could act intracellularly by inactivation of any BoNT that might escape the first line of defense.[215]

Analysis of the immune recognition of BoNTs has been limited to studies of their subunits or of relatively large fragments (50 kDa),[214,216,217] primarily because of the lack of structural information on these toxins. Recently, studies have emerged that aimed at narrowing down with synthetic peptides mAb recognition regions on the L chain.[218] In contrast, the immune recognition of TeNT has been investigated extensively,[219-223] mainly because of the earlier determination of its primary structure and the availability of human test samples. However, recent elucidation of the complete amino acid sequences of BoNTs has facilitated the mapping of the T- and B-cell (Ab) submolecular recognition of the BoNT molecules.

It has been shown that Abs against the receptor-binding regions on other bacterial toxins are very effective at neutralization of the correlate toxin. For example, the H_C-fragment of TeNT was shown to be a protective immunogen in mice against double the minimal lethal dose of TeNT.[223-225] In contrast, immunization of mice with a recombinant H_C of BoNT/A afforded protection against a high-challenge dose (10^5 LD_{50}) of BoNT/A.[226-228] The recombinant H_C fragment has also been microencapsulated in biodegradable poly-DL-lactide-co-glycoside microspheres,[229] and this antigen, when injected in mice, afforded 71% protection against aerosol challenge with BoNT/A.[229] Ten overlapping proteins were prepared by expression in *E. coli* of overlapping BoNT/A gene fragments, and of these only two (H455 to 661 and H1150 to 1289) were found to confer protection against BoNT/A poisoning.[230] Other studies have also suggested that H_C may have two receptor binding sites that are involved in BoNT internalization and toxicity[231] and whose blockage by mAbs might provide protection against BoNT/A toxicity.[231] Recently, five mAbs against

BoNT/E were shown to have BoNT/E-neutralizing activity in mice.[232] Three of these mAbs recognized regions around residues 663 to 668, 731 to 787, 811 to 897, respectively. Region 663 to 668 is close to the ion-channel-forming domain. The fourth mAb, which recognized a region close to the C-terminal part of H_C, might have interfered with BoNT-binding to the receptor on the target cell.[232]

A recombinant BoNT/C variant in which three amino acids were replaced (His229 → Gly, Glu230 → Thr, His233 → Asn) in the zinc-binding motif was found to be nontoxic to mice and did not cleave syntaxin in synaptosome preparations.[233] This recombinant neurotoxin stimulated high Ab levels and protective immunity when administered orally or subcutaneously.[232]

VII. MAPPING OF THE MOLECULAR AND CELLULAR IMMUNE RECOGNITION OF H_C

The finding that immunization with H_C of type A afforded excellent protection against BoNT/A poisoning[227,228] indicated that the immunological mapping of this region of BoNT/A would be extremely valuable for the eventual design of a synthetic peptide vaccine against BoNT. Therefore, we have performed a detailed mapping of the *continuous* regions of molecular and cellular immune recognition on the H_C region of BoNT/A (*continuous* regions are sites comprising residues that are directly linked by peptide bonds; *discontinuous* regions are sites comprising residues that are distant in sequence but come in close spatial proximity through folding of the polypeptide chain[234]). We employed a peptide-based strategy, previously developed in this laboratory,[235-238] for the localization of Ab and T-cell epitopes recognized by anti-BoNT/A and anti-H_C T and B cell responses. We also determined the peptides that, when used as immunogens, stimulated Ab and/or T-cell responses that cross-reacted with BoNT/A and/or with H_C. These peptides constitute most likely candidates for stimulation of active or passive (by Ab transfer) immunity against neurotoxin poisoning. It should be pointed out that, after localization of the protective regions on the H_C of BoNT/A, synthetic peptides can be made that correspond, on the other BoNT serotypes known to infect humans (most frequently types A, B, and E and rarely by type F), to the structural counterparts of the protective BoNT/A peptides. It has been well established that, on a set of homologous proteins, the regions of immune recognition occur on structurally equivalent locations.[239-242] Whereas peptide immunization has not generally provided useful protection against viral infections, it has proven to be quite effective against protein toxins.[243-246] Recent studies in this laboratory[246] have shown that immunization of mice with appropriate synthetic regions of α-bungarotoxin enabled the mice to survive a high α-bungarotoxin challenge dose (LD_{50} > 58 μg, when compared with an LD_{50} = 2.6 μg for nonimmunized mice), which was in fact higher than that obtained by immunization of mice with the whole toxin (LD_{50} = 9.69 μg).

For the epitope mapping, we prepared a panel of 31 synthetic consecutive overlapping peptides, of uniform size and overlaps, which encompassed the entire H_C polypeptide chain (residues 855 to 1296). The peptides were 19 residues each (except for peptide 31, which was 22 residues) and overlapped by 5 residues. The primary structures of the synthetic peptides are shown in Figure 2. It should be noted that this strategy is not designed to define the boundaries of the sites of immune recognition, but rather to obtain the locations within which these sites reside.[235-238]

A. H_C Regions Recognized by Anti-BoNT/A Antibodies from Three Outbred Host Species

The first step in the immunological mapping of the highly protective H_C domain of BoNT/A was done with anti-BoNT/A Abs that were prepared in outbred species.[247] Horse antisera were prepared by subcutaneous (s.c.) immunization in multiple sites, every 2 weeks for over 1 year, with a formaldehyde-inactivated BoNT/A in Ribi adjuvant. The serum tested in the binding studies was obtained after four injections.[247] Human antisera, which were made against the pentavalent toxoid (BoNTs A, B, C, D, and E) in human volunteers,[248] were obtained from Dr. John L. Middlebrook (Fort Detrick, Frederick, MD). The

Peptide	Residue Nos	Amino acid sequence
1	855-873	K Y V D N Q R L L S T F T E Y I K N I
2	869-887	Y I K N I I N T S I L N L R Y E S N H
3	883-901	Y E S N H L I D L S R Y A S K I N I G
4	897-915	K I N I G S K V N F D P I D K N Q I Q
5	911-929	K N Q I Q L F N L E S S K I E V I L K
6	925-943	E V I L K N A I V Y N S M Y E N F S T
7	939-957	E N F S T S F W I R I P K Y F N S I S
8	953-971	F N S I S L N N E Y T I I N C M E N N
9	967-985	C M E N N S G W K V S L N Y G E I I W
10	981-999	G E I I W T L Q D T Q E I K Q R V V F
11	995-1013	Q R V V F K Y S Q M I N I S D Y I N R
12	1009-1027	D Y I N R W I F V T I T N N R L N N S
13	1023-1041	R L N N S K I Y I N G R L I D Q K P I
14	1037-1055	D Q K P I S N L G N I H A S N N I M F
15	1051-1069	N N I M F K L D G C R D T H R Y I W I
16	1065-1083	R Y I W I K Y F N L F D K E L N E K E
17	1079-1097	L N E K E I K D L Y D N Q S N S G I L
18	1093-1111	N S G I L K D F W G D Y L Q Y D K P Y
19	1107-1125	Y D K P Y Y M L N L Y D P N K Y V D V
20	1121-1139	K Y V D V N N N V G I R G Y M Y L K G P
21	1135-1153	Y L K G P R G S V M T T N I Y L N S S
22	1149-1167	Y L N S S L Y R G T K F I I K K Y A S
23	1163-1181	K K Y A S G N K D N I V R N N D R V Y
24	1177-1195	N D R V Y I N V V V K N K E Y R L A T
25	1191-1209	Y R L A T N A S Q A G V E K I L S A L
26	1205-1223	I L S A L E I P D V G N L S Q V V V M
27	1219-1237	Q V V V M K S K N D Q G I T N K C K M
28	1233-1251	N K C K M N L Q D N N G N D I G F I G
29	1247-1265	I G F I G F H Q F N N I A K L V A S N
30	1261-1279	L V A S N W Y N R Q I E R S S R T L G
31	1275-1296	S R T L G C S W E F I P V D D G W G E R P L

FIGURE 2. Synthetic overlapping peptides of the protective H_C region of BoNT/A. The 31 peptides shown started at residue 855 and covered the entire sequence of H_C (residues 860 to 1296 of the H chain). Each peptide overlapped by five residues with each of its adjacent neighbors and the regions of overlap are shown in boldface type. (Figure is from Atassi et al.[247])

binding assays were done with the IgG fractions of these antisera.[247] Mouse anti-BoNT antisera, which were a pool from 20 mice obtained 91 days after the first injection,[247] were prepared in outbred ICR mice by s.c. immunization with toxoid.[247] For use as controls, nonimmune horse, and mouse sera were obtained from the corresponding animals before immunization, and nonimmune human IgG fraction was obtained from preimmune human sera.

Several regions of H_C were recognized by horse, human, and mouse anti-BoNT/A Abs. Comparison of the peptide binding profiles for horse, human, and mouse Abs revealed considerable similarities (see Figures 3, 4, and 5, and the summary in Table 1). Both human and mouse antisera recognized peptides 2 (residues 869 to 887), 15 (1051 to 1069), and 24 (1177 to 1195). With horse antiserum, both the first and second epitopes were shifted to the left and resided within peptides 1 (855 to 873) and 13/14 (1023 to 1041/1037 to 1055), respectively, while the third was shifted to the right and resided within the 25/26 (1191 to 1209/1205 to 1223) overlap. A region recognized by the human antisera within the overlap of peptides 5/6/7 (911 to 929/925 to 943/939 to 957) was more weakly recognized and shifted in favor of peptide 7 (939 to 957) in the mouse antisera. In horse antiserum, both peptides 5 (911 to 929) and 7 (939 to 957) (but not 6 [925 to 943]) were recognized. The lack of recognition of peptide 6 (925 to 943) suggests that this region harbors two epitopes that can be distinctly resolved by the horse, but not by the human and mouse, antisera with the present panel of peptides. The human antisera recognized a region within the overlap 10/11 (981 to 999/995 to 1013). This region was also recognized by mouse, and more weakly by horse, antisera and was shifted to the right toward peptide 11 (995 to 1013). Peptide 18 (1093 to 1111) was well recognized by horse, weakly by mouse, and not at all by human antisera. A very weak region was recognized by all three antisera around the overlap 20/21 (1121 to 1139/1135 to 1153) (human and mouse) or 20/21/22 (1121 to 1139/1135 to 1153/1149 to 1167) (horse). A broad region recognized within peptides 29/30/31 (1247 to 1265/1261 to 1279/1275 to 1296) by human antisera and within 30/31 (1261 to 1279/1275 to 1296) by horse antisera was more sharply localized within peptide 31 (1275 to 1296) by the mouse antisera. In addition to these shifts there were differences in immunodominance of the peptides recognized by antisera of the three species. It has been well established that the antigenic sites on a given protein may show boundary frame shifts and may also vary in immunodominance, depending on the host species in which the Abs are raised. These variations may even occur among individual animals of the same host species.[242,249,250] These results are consistent with genetic control operating at the antigenic site level. It is well established that the immune responses to proteins are controlled by H-2-linked genes[251-253] and that both B (i.e., Ab) and T cell responses to each epitope in a multideterminant protein antigen are under separate genetic control.[242,254-256]

B. The H_C Regions Recognized by Mouse Anti-BoNT/A Antibodies and T Cells

1. Binding of Antitoxoid Antibodies to the Overlapping Peptides

Mouse antisera were prepared against the pentavalent toxoid (BoNTs A, B, C, D, and E) in BALB/c ($H\text{-}2^d$) and SJL ($H\text{-}2^s$) mice and those collected on week 10 (i.e., 2 weeks after the last injection with toxoid) were employed for the binding studies to the peptides.[257] The binding profiles of antitoxoid Abs from BALB/c and SJL were quite similar (Figures 6 and 7). For BALB/c, antitoxoid Abs (Figure 6) bound mainly to peptides 24 (1177 to 1195), which was strongly immunodominant, the 2/3 (869 to 887/883 to 901) overlap, 21 (1135 to 1153) and 31 (1275 to 1296). In addition, lower but significant amounts of Abs were bound by peptides 11 (995 to 1013) and 15 (1051 to 1069). The other peptides exhibited marginal or no Ab binding activity. The antitoxoid Abs of SJL (Figure 7) recognized five antigenic regions within peptides 2/3 (869 to 887/883 to 901) overlap, 11 (995 to 1013), 15 (1051 to 1069), 24 (1177 to 1195), and 31 (1275 to 1296). Unlike BALB/c Abs, which exhibited low binding to peptides 11 (995 to 1013) and 15 (1051 to 1069), the Abs of SJL displayed high binding to both

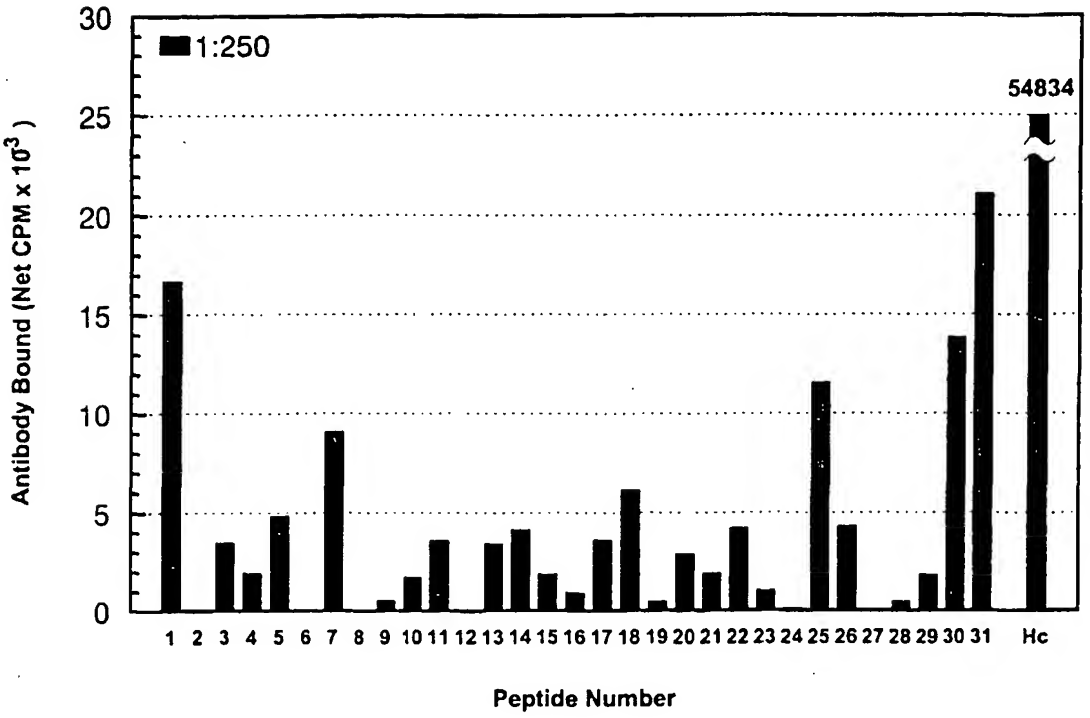

FIGURE 3. Binding of horse anti-BoNT/A antibodies to the overlapping BoNT/A peptides and to H_C. Binding was determined by solid-phase plate RIA using the antiserum at a dilution of 1:250 (vol/vol). The results were corrected for nonspecific binding of the antibodies to unrelated protein (BSA) and of preimmune sera to the peptides and to H_C. The data are expressed in net cpm and represent the average of triplicate analyses that varied ± 2.0% or less. (Figure is from Atassi et al.[247])

peptides. On the other hand, peptide 21 (1135 to 1153) bound higher amounts of BALB/c Abs than those of SJL.

2. Mapping of the T Cell Recognition Profiles

The profiles of *in vitro* responses to toxoid, mounted by T cells of BALB/c and SJL mice that had been primed with various doses of toxoid, were similar and gave the highest T cell response at a priming dose of 1 μg/mouse (e.g., see Figure 8 for BALB/c).[257] The results reviewed here were obtained with an optimal toxoid-priming dose of 1.0 μg/mouse for both mouse strains.[257] T cells of BALB/c mice, primed with one injection of toxoid, recognized two major regions localized within peptides 4 (residues 897 to 915) and 7 (939 to 957) (Figure 9). T cells of BALB/c, obtained after multiple inoculations with toxoid (i.e., at the time the hyperimmune antisera were obtained from the mice), showed an expanded recognition ability and responded very well to challenge with peptide 30 (1261 to 1279) and moderately to stimulation with peptide 22 (1149 to 1167). Unlike BALB/c T cells, those of toxoid-primed SJL exhibited a more complex profile and responded to challenge with a large number of overlapping peptides. After one toxoid injection, however, three regions within peptides 4 (897 to 915), 7/8 (939 to 957/953 to 971) overlap and 15 (1051 to 1069) were the most potent stimulators of T cells (Figure 10). After three toxoid injections (i.e., at the time the hyperimmune antitoxoid antisera were obtained from the SJL mice), peptides 4 (897 to 915) and 15 (1051 to 1069) remained immunodominant, while the third region

was shifted upstream and resided within the 6/7 (925 to 943/939 to 957) overlap. Peptides 4 (897 to 915) and 7 (939 to 957) were also recognized by BALB/c T cell (Figure 9). Table 2 compares the recognition profiles of antitoxoid Abs and T cells from the two strains.[257] The immunodominant epitope within peptide 4 (897 to 915) was recognized exclusively by T cells, because no Abs were detected against this region.

These findings (summarized in Table 2) show as expected[258] that, in a given strain, the regions recognized by antitoxoid Abs and T cells may coincide or may be uniquely B or T cell determinants. This is demonstrated by the following: (1) in a given strain, certain regions on H_C of BoNT/A were recognized by both Abs and T cells. Such T/B regions were identified within peptide 7 (939 to 957) in both strains. Additionally, SJL recognized three T/B regions located within peptides 15 (1051 to 1069), 24 (1177 to 1195), and 31 (1275 to 1296); (2) H_C contained regions that were recognized only by T cell, because no detectable Abs were directed toward these sites. One such exclusive T cell epitope, recognized in both mouse strains but particularly prominent in SJL mice, resided within peptide 4 (897 to 915); (3) finally, there were regions on H_C that were recognized only by Abs and for which no T cell responses were detected. Two exclusively B cell determinants, common for both strains, were found within regions 869 to 887/883 to 901 and 995 to 1013.

Comparison of the submolecular T cell recognition profiles of toxoid-primed LNC obtained from the two mouse strains revealed two distinct types of T cell epitopes on H_C. Some epitopes were unique for a given strain, while the others were recognized by toxoid-primed

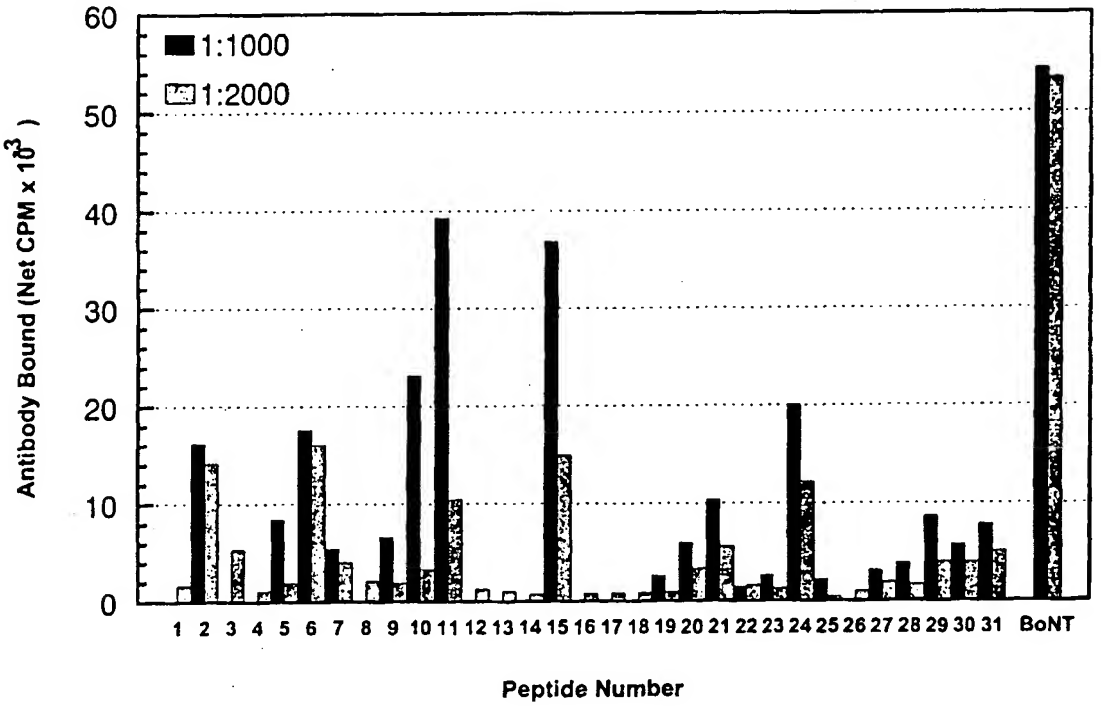

FIGURE 4. Binding of human antitoxoid antibodies to toxoid and to the overlapping peptides of the H_C domain of BoNT/A. Binding was done by solid-phase plate RIA at dilutions of 1:1000 and 1:2000 (vol/vol) of a 105 mg/ml solution of the IgG fraction of the antibody and has been corrected for nonspecific binding of the antibodies to an unrelated protein (BSA) and of nonimmune human IgG to the peptides and to BoNT/A. The results are given in net cpm of bound antibody and represent the average of triplicate analyses that varied ± 2.0% or less. (For details see the text. Figure is from Atassi et al.[247])

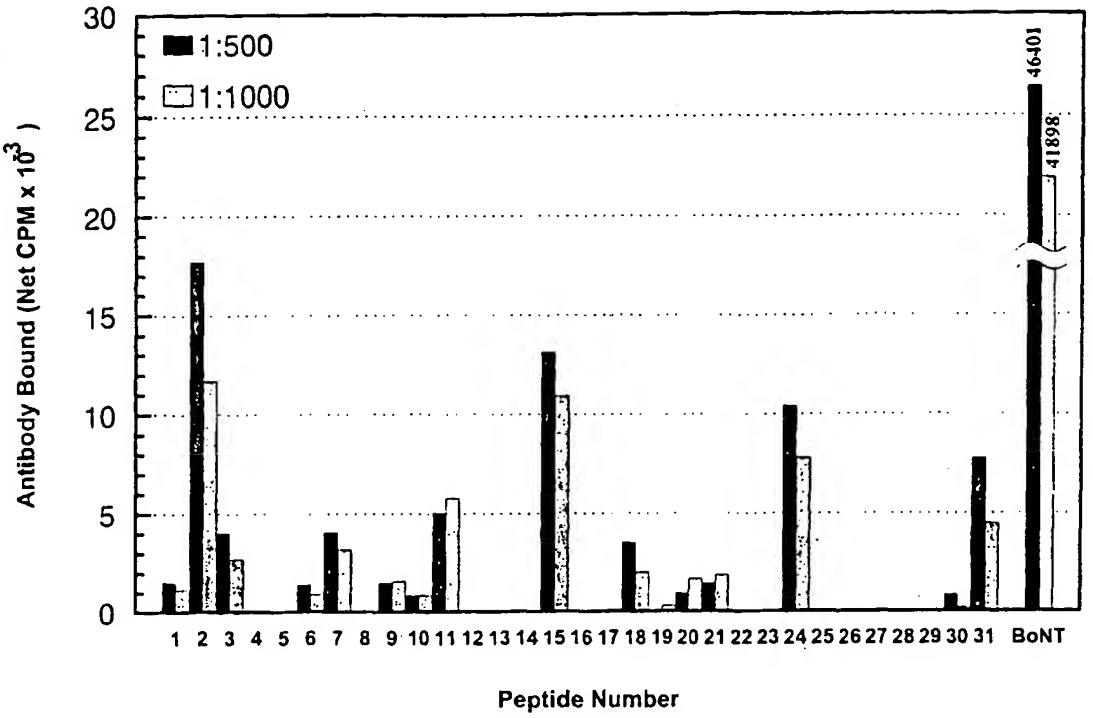

FIGURE 5. Binding of outbred (ICR) mouse antisera to the overlapping synthetic H_C peptides of BoNT/A. Binding was determined at two dilutions (1:500 and 1:1000, vol/vol) of the antisera and the results, which are expressed in net cpm, have been corrected for nonspecific binding of the antisera to unrelated protein (BSA) and by the preimmune sera to the toxoid and to each of the synthetic peptides. (For details, see the text. Figure is from Atassi et al.[247])

mice of both strains, irrespective of their MHC haplotype. In contrast to the T cell responses, the differences between the B cell recognition profiles of the two mouse haplotypes were less pronounced. Several regions recognized by Abs were similar, although the level of Abs to a given region varied with the strain (see Figures 6 and 7 or Table 2). Peptides that appear to be recognized across MHC haplotypes would be advantageous for a universal synthetic vaccine because they would be functional in many individuals. It is relevant to mention that the results presented here, which were obtained with toxoid-primed LNC (i.e., unselected Th cells)[257] may be more useful for the design of a synthetic vaccine than those derived from the best-growing T cell clones.[242,258–260]

C. Regions Recognized by Antibodies and/or by T Cells When H_C is Used as an Immunogen

The T cell responses of H_C-primed H-2^b, H-2^d, H-2^k, and H-2^s mouse haplotypes showed that SJL (H-2^s) and BALB/c (H-2^d) mouse strains are very high and high responders, respectively, to H_C.[261] These two mouse strains were used to map the *continuous* regions recognized by T-cell and Ab responses against H_C.[261] The synthetic over-

TABLE 1
Summary of Peptides Recognized by Horse Abs Against BoNT/A and Human and Mouse Abs Against Pentavalent Toxoid[a]

Pept. no.	Sequence position	Horse	Human	Mouse
1	855–873	+++	–	±
2	869–887	–	+++	+++
3	883–901	+	–	+
4	897–915	±	–	–
5	911–929	+	++	–
6	925–943	–	+++	–
7	939–957	++	+	+
8	953–971	–	–	–
9	967–985	–	+	±
10	981–999	±	+++	–
11	995–1013	+	+++++	+
12	1009–1027	–	–	–
13	1023–1041	+	–	–
14	1037–1055	+	–	–
15	1051–1069	±	+++++	++
16	1065–1083	–	–	–
17	1079–1097	+	–	–
18	1093–1111	+	–	+
19	1107–1125	–	±	–
20	1121–1139	+	+	±
21	1135–1153	±	++	±
22	1149–1167	+	–	–
23	1163–1181	–	±	–
24	1177–1195	–	+++	++
25	1191–1209	++	±	–
26	1205–1223	+	–	–
27	1219–1237	–	+	–
28	1233–1251	–	+	–
29	1247–1265	±	++	–
30	1261–1279	++	+	–
31	1275–1296	+++	++	++

[a] For the purpose of this table, (+) or (–) assignments were based on net cpm values, which, for human and mouse, were derived from the dilution that gave the highest binding. The symbols denote the following: (–), less than 1,500 cpm; (±), 1,500–3,000 cpm; (+), 3,000–7,000 cpm; (++), 7,000–15,000 cpm; (+++), 15,000–25,000 cpm; (++++), 25,000–35,000 cpm; (+++++), > 35,000 cpm.

Table is from Atassi et al.[247]

lapping peptides encompassing the entire H_C (residues 855 to 1296, Figure 2) were used for mapping the anti-H_C Ab and T cell responses.

1. Regions Recognized by Anti-H_C T Cells

In the H–2^s and H–2^d mouse haplotypes, the immunodominance in T cell recognition of various BoNT/A regions varied with the haplotype (Figure 11),[261] which is consistent with genetic control operating at the antigenic site level.[250-256,261] H_C-primed T cells of BALB/c recognized three regions residing within peptides 7 (residues 939 to 957), 12 (1009 to 1027), and 21 (1135 to 1153). The response to peptide 21 was immunodominant at 1 week (Figure 11) and persisted in long-term immunization.[261] The regions recognized strongly by T cells from H_C-primed SJL mice clustered in a large area within residues 897 to 985 comprising the overlapping peptides 4, 5, 6, 7, 8, and 9 in the first N-terminal third of H_C. There was only one additional region within peptide 15 (1051 to 1069), which stimulated a moderate response in these T cells.[261] The crowding of the regions recognized by SJL T cells to the first third of the H_C is unusual, and its significance (in terms of protection) in this strain needs further investigation. Dose-response curves[261] suggested that this cluster around peptides 4, 5, 6, 7, 8, and 9 might consist of at least two immunodominant epitopes around peptides 4 (897 to 915) and 7 (939 to 957). The immunodominance of region 939 to 957 persisted in hyperimmune T cells (long-term immunization). T cells of both SJL and BALB/c mice recognized region 939 to 957 (peptide 7) (Figure 11), indicating that this region of H_C can bind different MHC class II alleles. Promiscuous T cell epitopes that can be recognized by different MHC class II molecules might be beneficial for an universal vaccine, because human recipients of the vaccine possess different MHC class II haplotypes.

It has been reported[219] that in TeNT region 947 to 967 is recognized by human peripheral blood lymphocytes. This region of TeNT is homologous to BoNT/A region 938 to 958 within peptide 7 (residues 939 to 957), which is recognized by SJL and BALB/c T cells. Region 916 to 932 of TeNT (equivalent to BoNT/A region 907 to 923 within the overlap of peptides 4 [residues 897 to 915] and 5 [residues 911 to 929] recognized by SJL T cells[261]) has also been found to be recognized by human T cells.[221] These similarities in T-

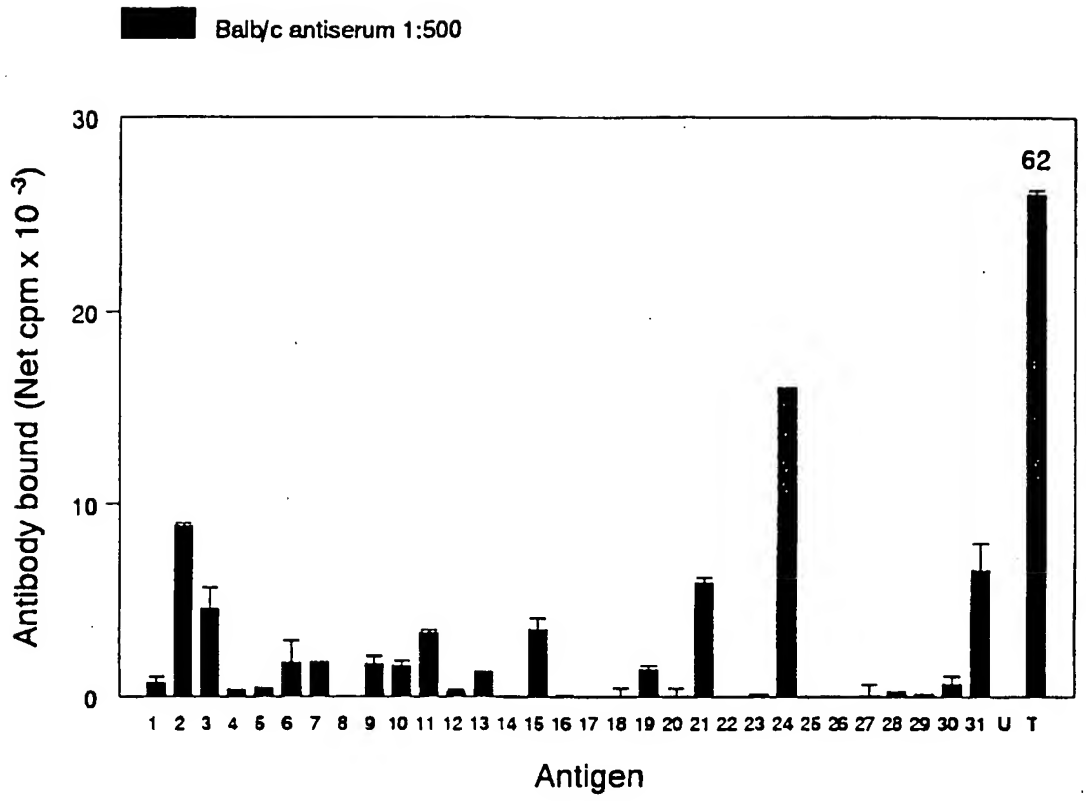

FIGURE 6. Binding of BALB/c antitoxoid Abs to toxoid (T), to the synthetic overlapping peptides of BoNT/A, and to the unrelated synthetic peptide (U) used as a negative control. For RIA, the antiserum was diluted 1:500 (v/v). Results are given in net cpm ± SD of triplicate analyses and have been corrected for nonspecific binding of the Abs to unrelated protein (BSA) and of the preimmune sera to the toxoid and to each of the synthetic peptides. The value on the top of bar designated T shows the amount of Abs (61,957 ± 778 net cpm) bound to the toxoid. (Figure is from Rosenberg et al.[257])

cell recognition regions indicate that the two clostridial toxins, BoNT and TeNT, share some of immunological features at the T cell level, along with a number of structural and functional similarities. As already mentioned, in closely related proteins, the sites of immune recognition often occur at structurally equivalent locations.[239,242]

2. The Regions Recognized by Anti-H_C Antibodies

While H_C-primed LNC from SJL and BALB/c recognized a common as well as different epitope regions on H_C, regions recognized by Abs from the two mouse strains essentially overlapped.[261] However, within a given antiserum, the active peptides bound different amounts of Abs (see Figures 12 and 13). Also, the levels of Abs bound by each region differed between the two strains (see Figure 13 and Table 3). There were seven common or similar regions (four common; three similar) of recognition in the two strains. Similar observations were made recently in SJL and C57BL/6 mice primed with AChR,[262] in which major Ab recognition regions for both strains were clustered into three similar regions within α1 to 210 of the AChR α chain, whereas T cells from each strain recognized different peptide regions.

Comparison of the profiles of the anti-H_C Ab and T-cell responses in the same mouse strains revealed, as seen above with the anti-BoNT/A

responses, that, in a given mouse strain certain regions are recognized by both Abs and T cells. There were also regions that were predominantly recognized only by Abs or only by T cells. It has been shown previously[237,242,244,262] that, in a given mouse strain, the regions on a protein that are recognized by Abs and by T cells may coincide, but the protein might also have regions that are recognized by Abs and for which T-cell responses are not detectable and/or conversely regions recognized by T-cells for which no Abs are detectable.

Peptides 2 to 11 (869–1013), 15 (1051–1069), 17 (1079–1097), 18 (1093–1111), 21 (1135–1153), 24 (1177–1195), and 31 (1275–1296) were well recognized (≥ ++, see Table 3) by Abs and/or by T cells from either strain.[261] Sequence alignment of these 16 peptide regions in BoNTs A through G and TeNT reveals[261] that 11 of the peptides have 5 or more continuous residues that are identical or similar to BoNT/A in one or more of these BoNTs (Figure 14). Although a five-residue homology (or similarity) may not be sufficient for cross-reaction (or cross-protection), it is probable that the epitopes in the other two BoNTs reside within these regions and that the H_C of each BoNT will be protective against the correlate toxin.

D. Reaction with H_C of Antibodies and T Cells Obtained After Immunization with Peptides

In order to understand the role of T cell and Ab recognition in cross-reaction with H_C and to devise an effective formula for a synthetic peptide

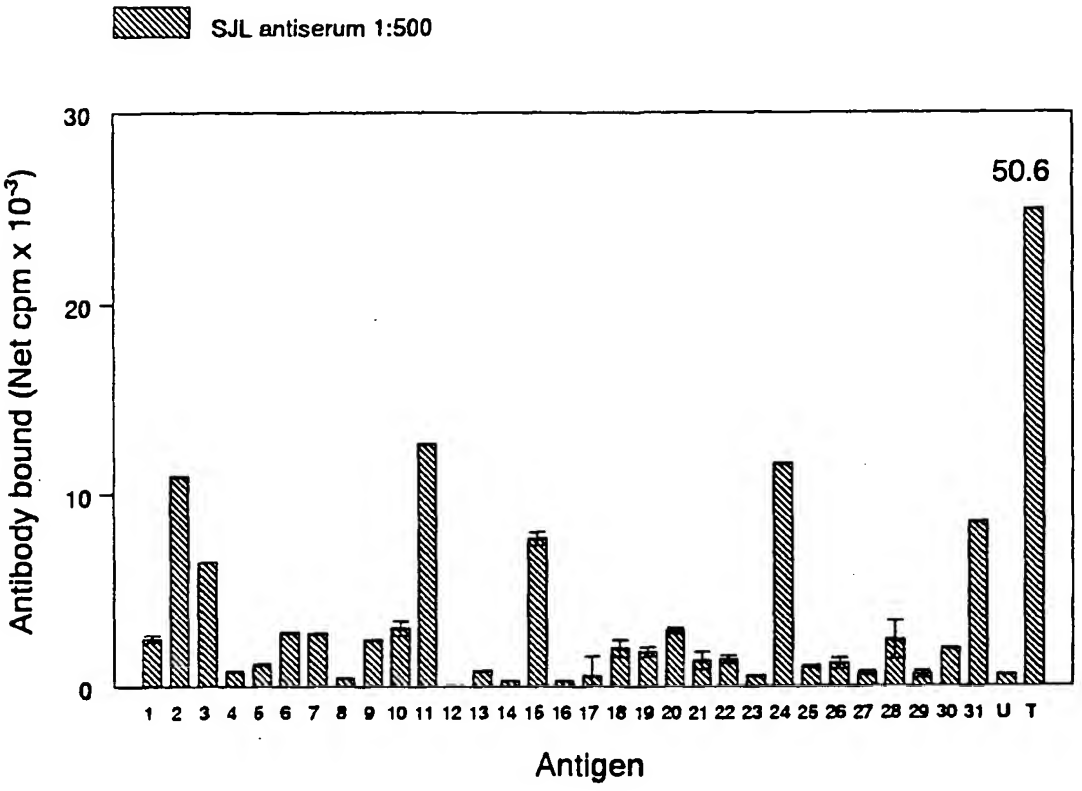

FIGURE 7. Binding of SJL antitoxoid Abs to toxoid (T), to the synthetic overlapping peptides (1 to 31) of BoNT/A, and to the unrelated peptide (U). Data were expressed and corrected as in Figure 6. Binding of antibodies to toxoid gave 50,575 ± 57 cpm as indicated on top of the bar T. (Figure is from Rosenberg et al.[257])

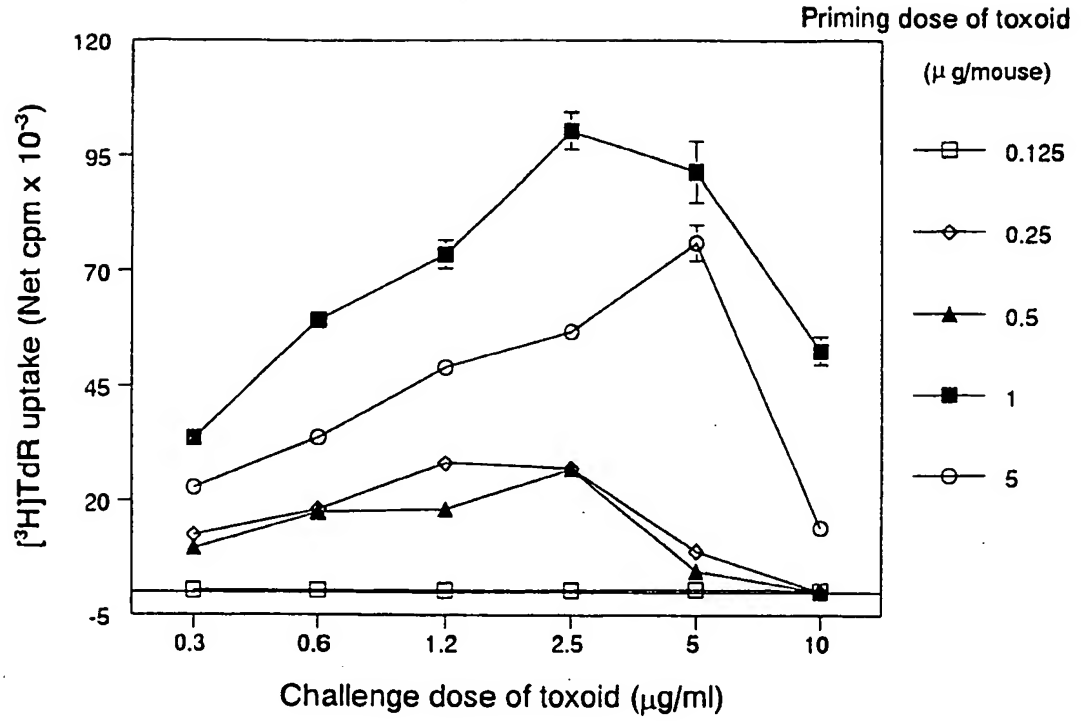

FIGURE 8. Dependence on the *in vitro* toxoid challenge dose of the proliferative response of LNC from BALB/c mice that were primed with different doses (0.125 to 5 μg/mouse) of pentavalent toxoid (BoNTs A, B, C, D, E). (Figure is from Rosenberg et al.[257])

vaccine, both Ab and T-cell responses against individual peptides and against mixtures of selected peptides need to be known. In the foregoing sections, we reviewed the immunodominant peptide regions on H_C that are recognized by T and/or B cells when either BoNT/A or H_C is used as an immunogen. Recently, these peptide regions were used as immunogens to determine those that stimulate immune responses that recognize intact H_C.[263] Three different mixtures of peptides were used as immunogens in two mouse strains (Table 4): (1) peptides containing epitopes recognized by anti-H_C T cells; (2) peptides containing epitopes recognized by anti-H_C B cells (Abs), or (3) peptides containing T cell + B cell epitopes.[263]

In BALB/c, all the peptides that contained Ab and/or T-cell epitopes (when H_C is the immunogen[261]) produced Ab responses against the immunizing peptide that cross-reacted with H_C. Strong H_C-cross-reactive Abs were generated[263] by peptides 2, 3, 10, and 31, which contain epitopes recognized by anti-H_C Abs[261] (Table 5). However, the levels of reaction with the immunizing peptides and with H_C varied. Among these, Abs against peptide 31 (residues 1275 to 1296) showed the highest binding to H_C. In SJL, antipeptide Abs were elicited by most of the peptides that contain Ab and T cell epitopes. The Ab responses were given, in decreasing order, by peptides 10, 4, 6, 7, 5, 8, 11, 24, 31, and 15.[263] However, very strong H_C-reactive anti-peptide Abs were elicited by peptide 4 (897 to 915) followed by peptide 10 (981 to 999). The greater immunogenicity of peptide 4 in SJL might be rationalized by the fact that it contains both T and B cell epitopes.[261,263] Thus, peptide 4 in SJL

and peptide 31 in BALB/c elicited Abs that gave the highest cross-reaction with H_C.

T cell responses against the individual peptides were also reported.[263] In BALB/c, the H_C-reactive anti-peptide T cells were those elicited by peptides 7 (939 to 957), 12 (1009 to 1027), and 17 (1079 to 1097). In SJL, T cell responses elicited by peptides 4 to 8 and 10 (981 to 999) were cross-reactive with H_C. Except for peptide 17 in BALB/c, each of these peptide-primed T cells showed moderate to very strong proliferative response to the immunizing peptide. However, T cells against peptides 2, 21, 24, and 31 in BALB/c and 15 and 31 in SJL showed negligible response to challenge with H_C (Table 5). Similar observations have been reported previously by this laboratory[258,264,265] and others[266,267] with anti-peptide T cells that failed to recognize the parent protein.

Equimolar mixtures of selected peptides were also employed as immunogens to stimulate Ab and T cell responses, and the cross-reactivity of these responses was determined with H_C and with each of the constituent peptides in the mixture.[263] Among the three groups of peptide mixtures, the one of peptides containing both Ab and T cell epitopes was most effective in both strains in eliciting T cells and Abs that were cross-reactive with H_C (Figure 15 shows binding of Abs to H_C). There

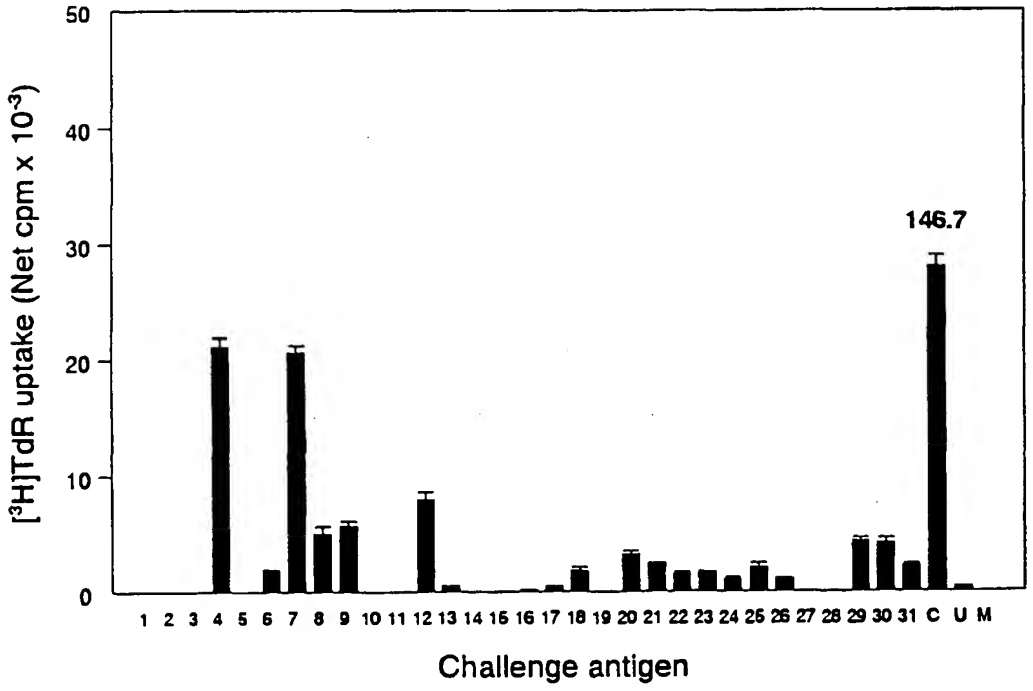

FIGURE 9. Proliferative response of LNC from toxoid-primed BALB/c mice to *in vitro* challenge with the synthetic overlapping BoNT/A peptides. Numbers 1 to 31 under the abscissa refer to the peptide numbers shown in Figure 2. Controls included H_C (C), unrelated synthetic peptide (U), and myoglobin (M). Results are expressed in net cpm ± SD of triplicate cultures at the optimal stimulation dose of each challenge antigen. The value on the top of the bar marked C indicates the vigorous T cell response to H_C (146,684 ± 1,801 cpm). The amount of [H^3]TdR incorporated by unstimulated cells was 6,166 ± 53 cpm. (Figure is from Rosenberg et al.[257])

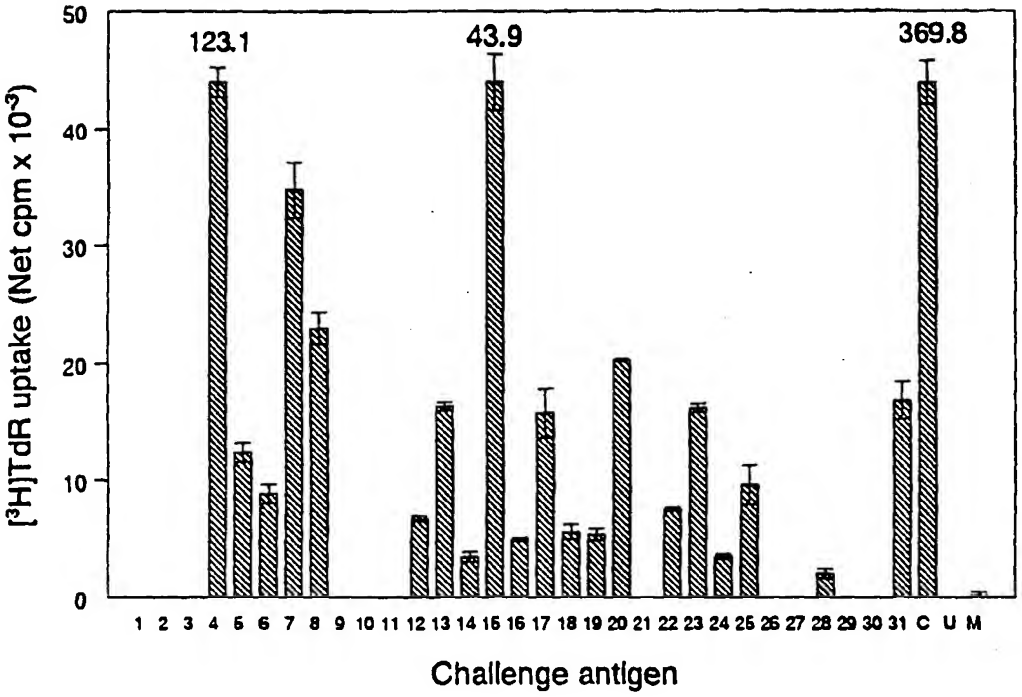

FIGURE 10. Proliferative response of LNC from toxoid-primed SJL mice to *in vitro* challenge with the synthetic overlapping peptides of BoNT/A. Numbers and symbols of the antigens are as in Figure 9. The values on top of the bars show the strong response of T cells to the challenge with peptide 4 (123,120 ± 1,219 cpm), peptide 15 (43,912 ± 3,335 cpm), and H_C (369,801 ± 1,800 cpm). The level of [H³]TdR incorporation in the absence of any antigenic stimulus was 12,331 ± 97 cpm. (Figure is from Rosenberg et al.[257])

were qualitative and quantitative differences in the peptide recognition profile after immunization with this peptide mixture when compared with those obtained with individual peptide immunization (Tables 5 and 6). Immunization with this mixture elicited Abs to some peptides that were otherwise unable to evoke Ab responses when used individually as immunogens (peptides 2, 3, and 9 in SJL). Also, it suppressed Ab responses to certain peptides that could otherwise elicit Abs when injected individually (peptides 12, 17, and 21 in BALB/c). Clearly, Ab production to these regions in the peptide mixture is modulated by help and inter-site influences of the cellular responses against the constituent peptides. It has been shown that immune responses to various epitopes on an antigen are subject to inter-site T-T and T-B cell interactions.[264,265,267–270] These interactions and co-immunization effects[264,265] contribute to the complex responses of T cells and Abs obtained after peptide mixture immunization.

Injection with the peptide mixture containing Ab and T cell epitope peptides (when H_C is the immunogen[261]) gave a quicker rise (after two injections, at 4 weeks) in Ab titer that cross-reacted with H_C compared with the other mixtures or to individual peptides.[263] Also, this mixture sustained a high titer of H_C-cross-reacting Abs in the case of BALB/c (Figure 15). Thus, immunization with a mixture of peptides containing all the T and B cell epitopes was particularly effective in BALB/c mice. The results suggest that inclusion of the peptides containing T cell epitopes into the vaccine formula should provide help for B cells that

TABLE 2
The Regions on the H_c Domain of BoNT/A That Are Recognized by Abs and/or T Cells After Immunization of BALB/c and SJL Mouse Strains with Toxoid[a]

Peptide	Position in sequence (residue numbers)	BALB/c (H-2^d) Ab	BALB/c (H-2^d) T cells	SJL (H-2^s) Ab	SJL (H-2^s) T cells
1	855–873	–	–	+	–
2	869–887	++	–	+++	–
3	883–901	++	–	++	–
4	897–915	–	++	–	++++
5	911–929	–	–	±	+
6	925–943	+	–	+	+
7	939–957	+	++	+	+++
8	953–971	–	–	–	++
9	967–985	+	–	+	–
10	981–999	+	–	+	–
11	995–1013	+	–	+++	–
12	1009–1027	–	+	–	+
13	1023–1041	±	–	–	++
14	1037–1055	–	–	–	+
15	1051–1069	+	–	++	+++
16	1065–1083	–	–	–	+
17	1079–1097	–	–	–	++
18	1093–1111	–	–	+	+
19	1107–1125	±	–	+	+
20	1121–1139	–	–	+	++
21	1135–1153	++	–	±	+
22	1149–1167	–	–	±	+
23	1163–1181	–	–	–	++
24	1177–1195	+++	–	+++	+
25	1191–1209	–	–	+	+
26	1205–1223	–	–	±	–
27	1219–1237	–	–	–	–
28	1233–1251	–	–	+	+
29	1247–1265	–	–	–	+
30	1261–1279	–	–	+	–
31	1275–1296	++	–	++	++

[a] For the purpose of this table, (+) and (–) assignments were based on net cpm values for Ab binding and SI values for T cell proliferation. For Ab binding, the symbols denote the following values: (–), less than 1000 cpm; (±), 1001–1500 cpm; (+), 1501–4000 cpm; (++), 4001–10,000 cpm; (+++), > 10,000. For T cell proliferation, the symbols indicate the following: (–); SI value less than 2.0; (+), SI 2.0–3.5; (++), SI 3.6–4.5; (+++), SI 4.6–10.0; (++++), SI > 10.0. Results of T and B cell mapping studies were obtained with mice that received single and multiple injections, respectively.

Table is from Rosenberg et al.[257]

make H_c-cross-reactive Abs and thus enhance the production of these Abs. Recently, it has been shown that a mixture of three peptides from α-bungarotoxin was a more protective immunogen against toxin poisoning than any of the peptides constituting the mixture when used individually.[246]

Sequence alignment of BoNT types A through G and TeNT in the 17 peptide regions used as

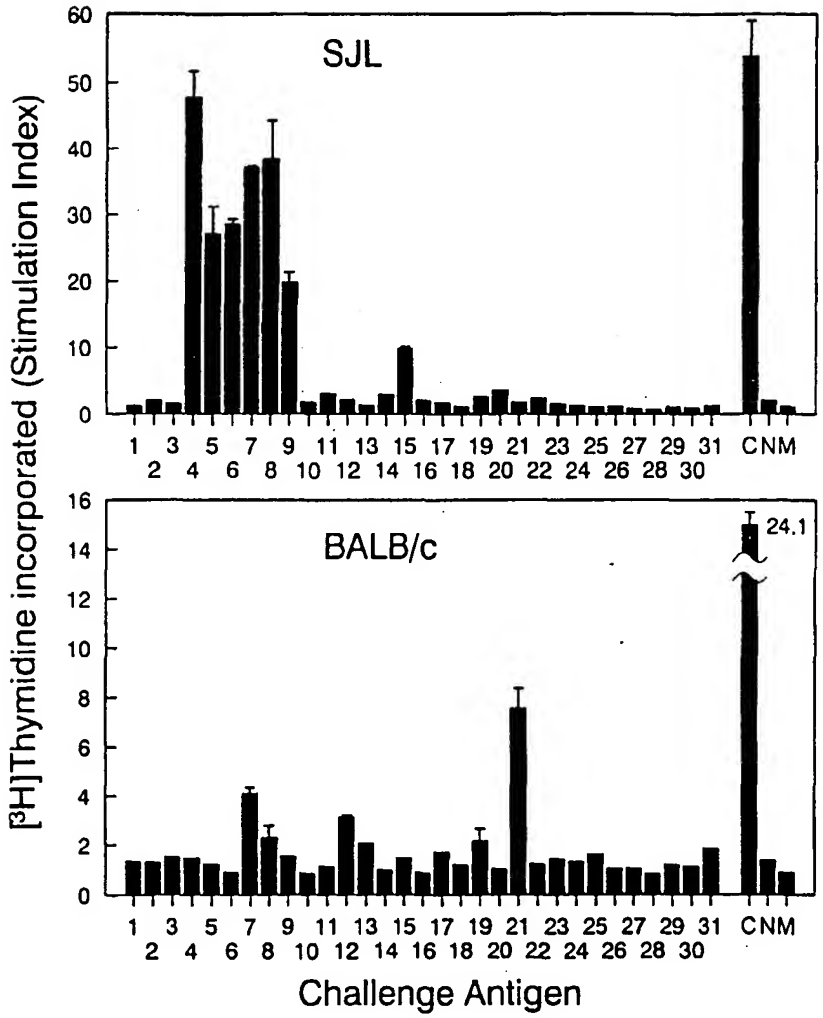

FIGURE 11. Presentation showing the *in vitro* proliferative response to the BoNT/A peptides 1 to 31 of LNC from SJL mice and from BALB/c mice primed with 0.25 µg/mouse of H_C. The diagram shows S.I. at the optimum challenge doses of each peptide and H_C. Unstimulated cells gave 2,330 ± 168 cpm for SJL and 3,534 ± 141 cpm for BALB/c. Numbers 1 to 31 refer to the peptides shown in Table 1. Additional antigen letter symbols are C, H_C; N, unrelated synthetic peptide; M, myoglobin. (Figure is from Oshima et al.[261])

immunogens revealed[263] that 13 peptides have, in one or more of these clostridial toxins, five or more continuous residues that are identical or similar to BoNT/A (Figure 14). Of these, peptides 2, 3, 7, 10, 12, 15, 18, 24, and 31 were shown to generate Abs that are cross-reactive with H_C in either strain (Table 5). Addition of peptides 7 (residues 939 to 957) and 12 (1009 to 1027), which contain T cell epitopes and have identical or similar regions in most of the clostridial toxins, to the mixture that consisted of peptides containing Ab epitopes augmented production of Abs

that are cross-reactive with H_C in BALB/c (Figure 15). These results suggest that one or more of the synthetic peptides provide help that might contribute to cross-protection against those toxins. Peptide 7 (939 to 957), a T and/or Ab epitope-containing peptide for both strains, is immunogenic at both the T and B cell levels in each strain when used as immunogen either individually or in a mixture (Tables 5 and 6). It also generated T cell and Ab responses that were cross-reactive with H_C (Table 5). It should be noted that region 947 to 967 of TeNT, similar region to peptide 7 (residues 939 to 957), is also a universal human T cell epitope region for TeNT.[270] The fact that peptide 7 is effective in both strains suggests that it needs to be included in the design of synthetic vaccines that will be active across MHC haplotypes.

VIII. CONCLUSIONS

The epitopes on the protective H_C region of BoNT/A (residues 855 to 1296), which are recognized by anti-BoNT/A, have been mapped with Abs raised in horse, human, and mouse. Using two mouse strains [BALB/c ($H\text{-}2^d$) and SJL ($H\text{-}2^s$)], the epitopes on the H_C that are recognized by anti-

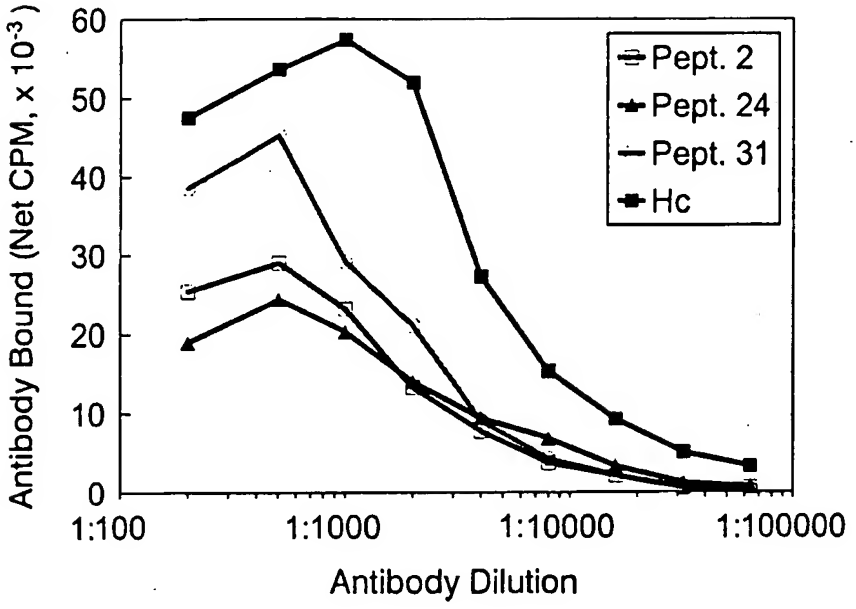

FIGURE 12. Binding of anti-H_C Abs (BALB/c, at 12 weeks) to H_C and three selected BoNT/A peptides. Different dilutions (from 1:200 to 1:64000; v/v) of anti-H_C antisera were assayed by solid-phase RIA. Preimmune sera were used as a negative control, and their values were subtracted to obtain the net cpm. (Figure is from Oshima et al.[261])

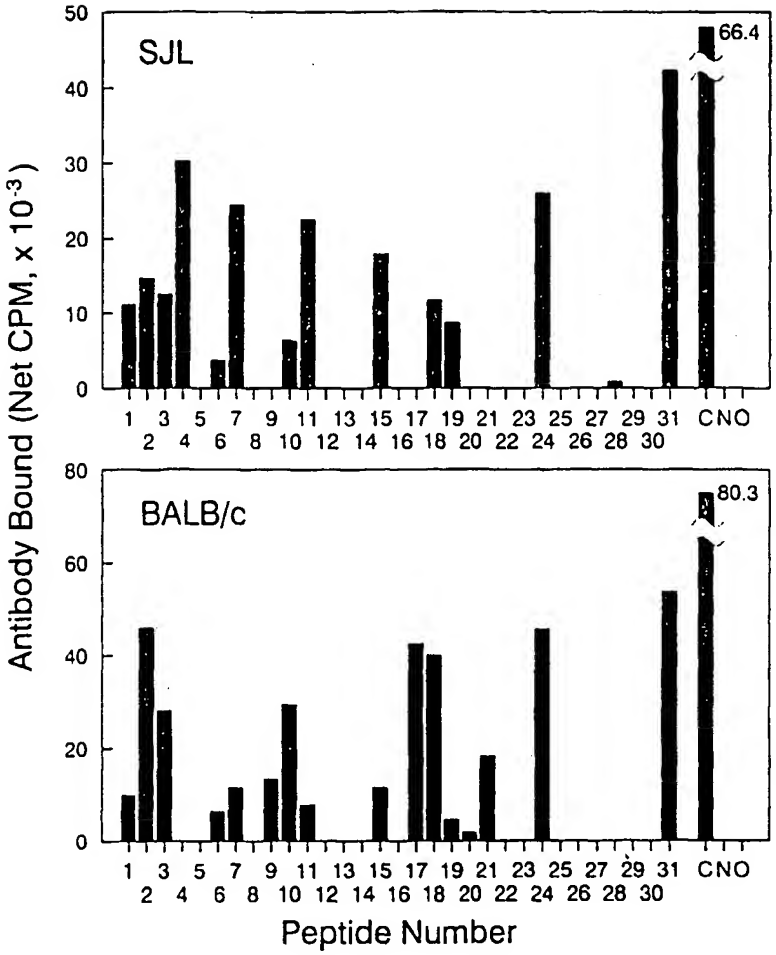

FIGURE 13. Ab binding to the synthetic BoNT/A peptides of antisera from SJL and BALB/c after four immunizations (12 weeks after initial injection) with H_C (SJL, 0.5 µg/mouse; BALB/c, 0.25 µg/mouse). The diagram shows the net cpm in which the average binding value of the same antigen to the preimmune sera was subtracted. Numbers 1 to 31 refer to the peptides shown in Table 1. Additional antigen letter symbols are C, H_C; N, unrelated synthetic peptide; O, ovalbumin. (Figure is from Oshima et al.[261])

H_C Abs and by H_C-primed T lymphocytes were mapped. The peptides, which contain Ab or T-cell epitopes (or both) on the H_C, were used as immunogens in BALB/c and SJL mice and we identified those peptides whose Ab and/or T-cell responses cross-react with H_C. Identification of these peptides is an important first step in the intricate requirements for the design of a synthetic vaccine.

ACKNOWLEDGMENTS

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TABLE 3
Summary of Peptides Recognized by Abs and by T Lymphocytes When H_c is Used as an Immunogen in SJL and BALB/c Mouse Strains[a]

Peptide number	Sequence position	SJL ($H\text{-}2^s$)		BALB/c ($H\text{-}2^d$)	
		Ab	T cells	Ab	T cells
1	855–873	+	–	+	–
2	869–887	++	–	++++	–
3	883–901	++	–	+++	–
4	897–915	+++	++++	–	–
5	911–929	–	+++	–	–
6	925–943	±	+++	+	–
7	939–957	+++	++++	+	+
8	953–971	–	++++	–	±
9	967–985	–	+++	++	–
10	981–999	+	–	+++	–
11	995–1013	+++	+	+	–
12	1009–1027	–	±	–	+
13	1023–1041	–	–	–	±
14	1037–1055	–	+	–	–
15	1051–1069	++	++	+	–
16	1065–1083	–	±	–	–
17	1079–1097	–	–	++++	–
18	1093–1111	+	–	++++	–
19	1107–1125	+	±	±	±
20	1121–1139	–	+	–	–
21	1135–1153	–	–	++	++
22	1149–1167	–	±	–	–
23	1163–1181	–	–	–	–
24	1177–1195	+++	–	++++	–
25	1191–1209	–	–	–	–
26	1205–1223	–	–	–	–
27	1219–1237	–	–	–	–
28	1233–1251	–	–	–	–
29	1247–1265	–	–	–	–
30	1261–1279	–	–	–	–
31	1275–1296	++++	–	++++	–

[a] Assignment of positive and negative responses for the purpose of this table was based on net cpm values for Ab study and of S.I. values for T-cell study. For Ab binding, the symbols denote the following values: (–), less than 2000 cpm; (±), 2000–5000 cpm; (+), 5000–12,000 cpm; (++), 12,000–22,000 cpm; (+++), 22,000–40,000 cpm; (++++), > 40,000 cpm. For T cell recognition, the symbols denote the following: (–), S.I. value less than 2.0; (±), S.I. 2.0–2.9; (+), S.I. 3.0–4.9; (++), S.I. 5.0–9.9; (+++), S.I. 10.0–29.9; (++++), S.I. ≥ 30.0.

Table is from Oshima et al.[261]

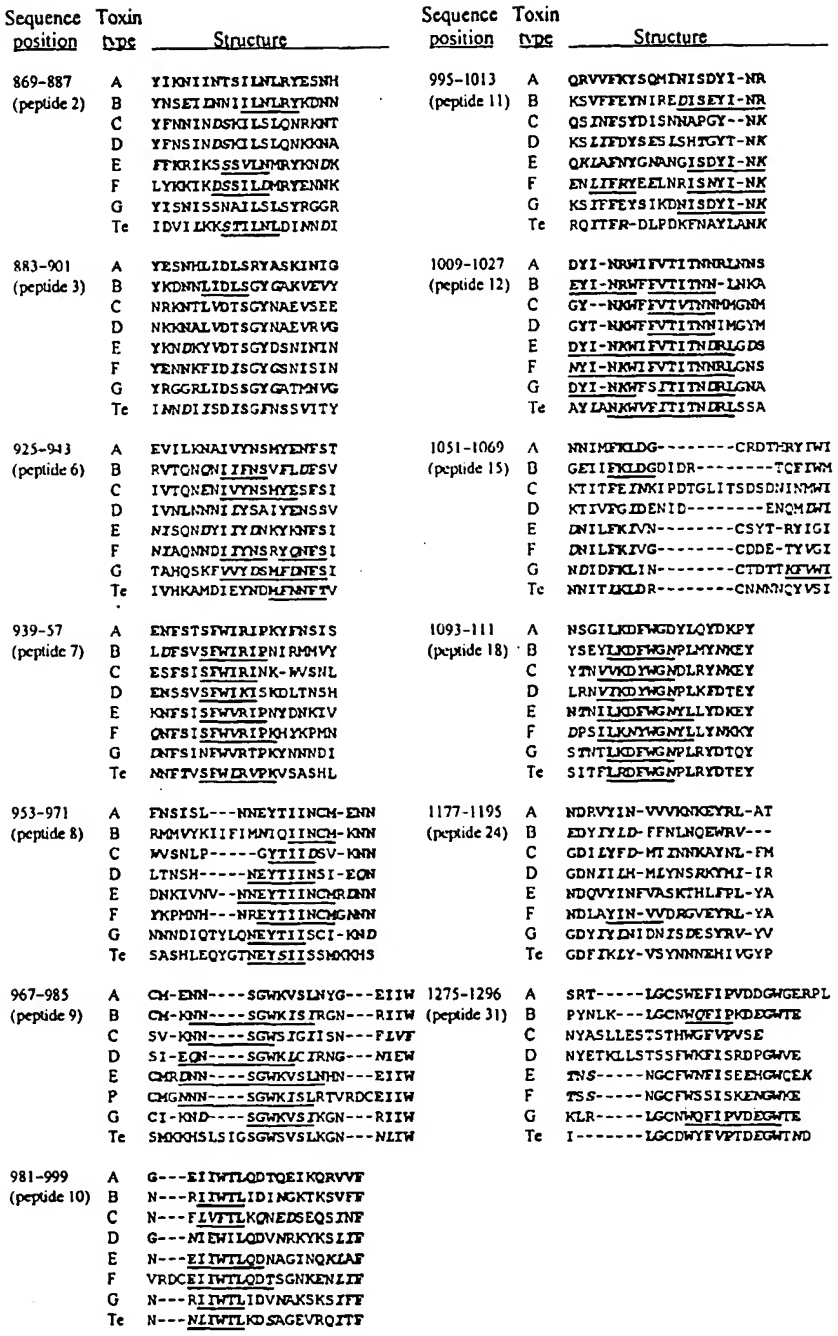

FIGURE 14. Comparative alignment of BoNT types A through G and TeNT within 13 peptide regions on H_C. Alignment is from Whelan et al.[271] Boldface letter in BoNT/A signify residues that are identical or similar to the amino acid in one or more of the toxin types listed. In BoNTs B through G, residues identical to those of BoNT/A are in boldface type. Boldface and italic letters represent the residues in which conservative replacements have occurred. Regions that have five or more continuous residues identical or similar to BoNT/A sequence are underlined. (Figure is adapted with expansion from Oshima et al.[261])

TABLE 4
Constituent Peptides of Peptide Mixtures Containing T Cell and/or Ab Epitopes

Peptide mixture	SJL	BALB/c
T Cell	Peptides 4–9 and 15	Peptides 7, 12, and 21
Ab	Peptides 2–4, 7, 10, 11, 15, 24, and 31	Peptides 2, 3, 10, 17, 18, 21, 24, and 31
T Cell + Ab	Peptides 2–11, 15, 24, and 31	Peptides 2, 3, 7, 10, 12, 17, 18, 21, 24, and 31

Table is from Oshima et al.[263]

TABLE 5
Summary of Reaction with Immunizing Peptide and H_c of Immune Responses Elicited When Individual BoNT/A Peptides, Including T and/or Ab Epitope, Are Used as Immunogens in SJL and BALB/c Mouse Strains[a]

Peptide immunogen		SJL ($H\text{-}2^s$)					BALB/c ($H\text{-}2^d$)				
			Ab		T cell			Ab		T cell	
Pept. no.	Sequence position	Epitope for[b]	Pept.	H_c	Pept.	H_c	Epitope for[b]	Pept.	H_c	Pept.	H_c
2	869–887	Ab	±	–	–	–	Ab	++++	++++	+++	–
3	883–901	Ab	–	–	±	–	Ab	++	++++	±	–
4	897–915	T, Ab	+++++	+++++	+++++	++++	n/e[c]	n/e	n/e	n/e	n/e
5	911–929	T	++++	+	++++	++++	n/e	n/e	n/e	n/e	n/e
6	925–943	T	+++++	–	+++	+++	n/e	n/e	n/e	n/e	n/e
7	939–957	T, Ab	+++++	+	+++++	++++	T	+++++	++	+++	+++
8	953–971	T	+++	–	++++	++++	n/e	n/e	n/e	n/e	n/e
9	967–985	T	–	–	±	–	n/e	n/e	n/e	n/e	n/e
10	981–999	Ab	+++++	++	+++	+	Ab	++++	++++	–	–
11	995–1013	Ab	++	±	–	–	n/e	n/e	n/e	n/e	n/e
12	1009–1027	n/e	n/e	n/e	n/e	n/e	T	+++++	+++	+++	+
15	1051–1069	T, Ab	+	+	+++	±	n/e	n/e	n/e	n/e	n/e
17	1079–1097	n/e	n/e	n/e	n/e	n/e	Ab	+++	+++	±	+
18	1093–1111	n/e	n/e	n/e	n/e	n/e	Ab	++	++	–	–
21	1135–1153	n/e	n/e	n/e	n/e	n/e	T, Ab	+++	+++	+++	–
24	1177–1195	Ab	++	+	±	–	Ab	+++	+++	++	–
31	1275–1296	Ab	+	+	++	–	Ab	++++	++++	++	–

[a] Assignment of positive and negative responses for the purpose of this table was based on net cpm values for Ab study and of S.I. values for the T-cell study. For Ab binding, the symbols denote the following values: (–), less than 2000 cpm; (±), 2000–5000 cpm; (+), 5000–12,000 cpm; (++), 12,000–22,000 cpm; (+++), 22,000–40,000 cpm; (++++), 40,000–60,000 cpm; (+++++), > 60,000 cpm. For T cell recognition, the symbols denote the following: (–), S.I. value less than 2.0; (±), S.I. 2.0–2.9; (+), S.I. 3.0–5.0; (++), S.I. 5.1–10.0; (+++), S.I. 10.1–30.0; (++++), S.I. 30.1–60.0; (+++++), S.I. ≥ 60.1. All the anti-peptide antisera were unresponsive to protein and peptide controls used. The LNC of all experiments were unresponsive to unrelated proteins or peptide but responded appropriately to Con A and LPS.

[b] When H_c is used as the immunogen.

[c] n/e: indicates that the peptide is neither an Ab nor a T-cell epitope in this mouse strain when it is immunized with H_c, and therefore the peptide was not used as an immunogen in this mouse strain.

Results in this table are summarized from the data reported by Oshima et al.[263]

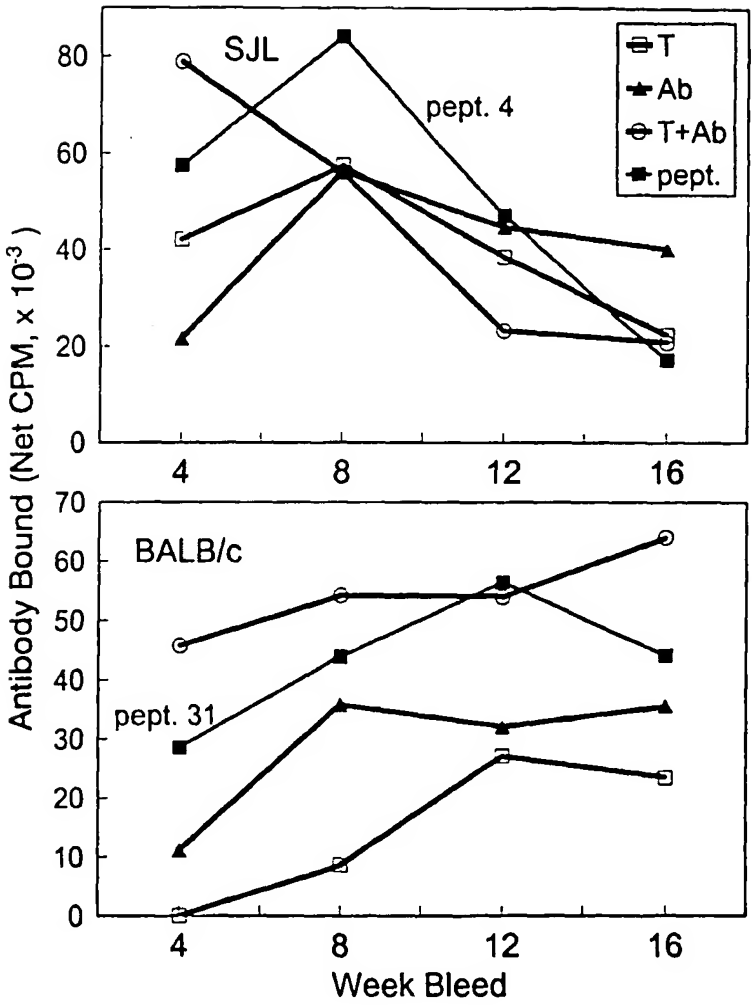

FIGURE 15. Binding to H_c of Abs against three peptide mixtures or against peptide 4 (SJL) and peptide 31 (BALB/c) obtained at 4, 8, 12, and 16 weeks. SJL and BALB/c mice were immunized with an equimolar mixture of peptides containing T cell and/or Ab epitopes (when H_c is the immunogen[261]) or with individual peptides at 0, 3, 7, 11, and 15 weeks. Preimmune sera were used as negative controls, and their values were subtracted to obtain the net cpm. For details see text. "T", "Ab", and "T + Ab" represent the mixture of peptides containing T cell epitopes, mixture of peptides containing Ab epitopes, and mixture of peptides containing both T cell and Ab epitopes, respectively. For the constituent peptides of each T cell and/or Ab epitope peptide mixtures, see Table 4. (Figure is from Oshima et al.[263])

TABLE 6
Summary of Immune Responses Elicited When an Equimolar Mixture of Peptides Containing T and B Cell Epitopes Was Used as Immunogens in SJL and BALB/c[a]

Pept. no.	SJL ($H\text{-}2^s$)		BALB/c ($H\text{-}2^d$)	
	Ab	T cell	Ab	T cell
2	++	–	+++	±
3	+++	+++	+++	–
4	+++++	++++	n/e[b]	n/e
5	±	++	n/e	n/e
6	+	+++	n/e	n/e
7	+++	++++	+++	+++
8	+++	++	n/e	n/e
9	+++	+++	n/e	n/e
10	++++	+++	+++	–
11	+	–	n/e	n/e
12	n/e	n/e	–	+++
15	±	+++	n/e	n/e
17	n/e	n/e	–	–
18	n/e	n/e	++	–
21	n/e	n/e	–	++
24	++	++	+++	±
31	+++	+++	++++	–
H_c	+++++	+++++	+++++	+++

[a,b] See footnotes for Table 5.

Results in this table are summarized from the report by Oshima et al.[263]

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